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COMPOSITION OF COMMERCIAL ACID LEAD ARSENATE AND ITS RELATION TO ARSENICAL INJURY[1]

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INTRODUCTION

Since acid lead arsenate was first used as an insecticide in 1892, its manufacture has become relatively well stabilized and a fairly uniform product is assured. Analyses of a considerable number of samples over a period of several years, however, show that more or less variation occurs in the quantities of arsenious oxide, total arsenic, and water-soluble arsenic present.

The total proportion of arsenic theoretically present in pure acid lead arsenate, expressed as arsenic pentoxide, is 33.1 per cent. Actually it was found to range from 28.0 to 32.8 per cent, thus indicating that commercial acid lead arsenate contains more or less basic lead arsenate. The more of the basic form present the safer the material is on foliage and the less toxic it is to insects (*3, p. 50; 6; 9; 10*).[2]

Although arsenious oxide should theoretically be absent, it was found to be present in quantities ranging from 0.16 to 1.40 per cent. As far as the writer is aware, the relation of the presence of this material to foliage injury has not been previously investigated.

The content of the water-soluble arsenic ranged from 0.04 to 5.93 per cent as arsenic pentoxide. The maximum guaranteed ranges from 0.38 to 1.15 per cent, although as a rule manufacturers guarantee their product to contain not more than 0.75 per cent. Little information has been available as to the effect of these quantities of water-soluble arsenic and the necessary minimum to avoid foliage injury. The quantity of soluble arsenic pentoxide present is regarded by some as of little importance so long as it is below the 0.75 per cent maximum set for lead arsenate paste by the insecticide act of 1910, which has been rather generally adopted by manufacturers for the powdered form now used. Others consider it of great importance, extravagant claims often being made by manufacturers and salesmen for a particular brand of lead arsenate as noninjurious to foliage because of its low soluble-arsenic content.

A series of experiments was conducted to determine the effect of these variations in composition upon foliage injury. All spraying tests were conducted upon peach trees, which are perhaps the most sensitive to arsenical injury of all the plants to which acid lead arsenate is extensively applied. These tests were carried on from 1926 to 1928 in central Georgia.

[1] Received for publication Feb. 26, 1929; issued September, 1929.

[2] Reference is made by number (italic) to "Literature cited," p. 400.

TOXIC CONCENTRATIONS OF ARSENIOUS AND ARSENIC ACIDS

To determine the minimum concentration of soluble arsenic toxic to peach foliage, solutions of arsenious and arsenic acids were made up in concentrations ranging from 0.0001 to 3.0 per cent expressed as arsenic pentoxide.[3] These were applied to peach trees with a hand sprayer until the spray material dripped from the foliage, and the results were noted daily. A summary of the results of these tests is given in Table 1.

TABLE 1.—*Effect of arsenious and arsenic acids, in various concentrations, upon peach foliage*

Concentration of acid used (as As_2O_5)	H-ion concentrations [a]		Time from spraying to first injury		Final effect on foliage	
	As_2O_3	As_2O_5	As_2O_3	As_2O_5	As_2O_3	As_2O_5
Per cent	*pH*	*pH*	*Days*	*Days*		
0.0001	6.4	6.3	------	------	No injury	No injury.
.0005	6.4	6.1	------	------	....do	Do.
.0010	6.4	5.9	------	------	....do	Do.
.0015	6.35	5.5	8	8	Light leaf burning	Light leaf burning.
.0020	6.35	5.1	8	8	....do	Do.
.0025	6.35	4.6	5	5	Moderate leaf burning	Moderate leaf burning.
.0050	6.35	3.5	5	5	Severe leaf burning	Severe leaf burning.
.0075	6.3	3.2	5	5	Severe leaf burning, light twig cankering, moderate defoliation.	Severe leaf burning, light twig cankering, light defoliation.
.0100	6.3	2.6	5	3	....do	Do.
.0200	6.3	2.5	5	3	Severe defoliation, light cankering.	Severe defoliation, severe cankering.
.0300	6.3	2.5	5	2	Severe defoliation, severe cankering.	Complete defoliation, very severe cankering.
.0400	6.25	2.45	5	2	Complete defoliation, severe cankering.	All twigs killed.
.0500	6.2	2.4	5	2	....do	Do.
.1000	5.4	2.2	3	2	All twigs killed	Do.
.2000	5.3	2.1	3	1	....do	Do.
.3000	5.2	2.0	3	1	....do	Do.
.4000	5.1	1.9	2	1	....do	Do.
.5000	5.0	1.9	1	1	....do	Do.
.6000	4.9	1.85	1	1	....do	Do.
.7000	4.85	1.8	1	1	....do	Do.
.8000	4.8	1.75	1	1	....do	Do.
.9000	4.7	1.75	1	1	....do	Do.
1.0000	4.6	1.6	1	1	....do	Do.
1.5000	3.8	1.5	1	1	....do	Do.
2.0000	3.6	1.5	1	1	....do	Do.
2.5000	3.4	1.5	1	1	....do	Do.
3.0000	3.3	1.5	1	1	....do	Do.

[a] Hydrogen-ion concentration of water used, pH 6.5.

DISCUSSION OF RESULTS

At low concentrations (below 0.01 per cent as arsenic pentoxide) the arsenious and arsenic acids were of equal toxicity when compared upon the basis of metallic arsenic content. Upon the same basis, at high concentrations arsenic acid was the more toxic, possibly because of its greater penetrative power due to its higher acidity. This difference in toxicity occurs at acidities of arsenic acid of pH 2.6 and higher.

No difference in toxicity between arsenious and arsenic acids was apparent at the concentrations at which either is present as soluble arsenic in lead arsenate.

[3] The percentages are based upon grams of solute per hundred grams of solution.

The minimum concentration toxic to peach leaves contained the equivalent of 0.0015 per cent arsenic pentoxide.

TESTS OF ARSENIC AS A CUMULATIVE POISON

As the arsenic upon a leaf can be in solution only when water is present, its action is more or less intermittent. To determine the minimum toxic concentration under these conditions and to ascertain whether arsenic might act as a cumulative poison, the experiments reported in Table 2 were performed. Concentrations under the toxic dosage of 0.0015 per cent arsenic pentoxide were applied successively to the same foliage and the results noted. A record of the rainfall during this period is given in Table 3. On June 30 it began to rain nine hours after the sprays had been applied, and on July 5, five hours afterwards. On July 13 and 16 spraying was begun approximately one hour after it had ceased raining.

TABLE 2.—*The cumulative effect of repeated applications of arsenic acid, in various concentrations, to peach foliage from June 29 to July 22, 1928*

Plot No.	Percentage of As_2O_5 used	Dates sprayed	Effect on foliage
1	0.0003	June 29	No injury.
2	.0003	June 29, 30	Do.
3	.0003	June 29, 30, July 2	Do.
4	.0003	June 29, 30, July 2, 3	Very light burning of leaves.
5	.0003	June 29, 30, July 2, 3, 5	Moderate burning of leaves.
6	.0015	June 29	Light burning of leaves.
7	.0003	June 29, 30	Do.
	.0009	July 13	
8	.0009	do	No injury.
9	.0008	July 16, 19	Moderate burning of leaves.
10	.0008	July 16, 22	Do.
11	.0008	July 16	No injury.

TABLE 3.—*Rainfall during the period of the tests of Table 2, June 29 to July 22, 1928*

Date	Rainfall	Date	Rainfall
	Inches		*Inches*
June 30	Trace.	July 12	1.13
July 5	0.68	July 13	2.80
July 6	.33	July 16	2.00
July 9	.43	July 17	.31
July 10	.04	July 18	Trace.
July 11	.50	July 21	.40

DISCUSSION OF RESULTS

The results on plots 1 to 6 showed that slight injury could occur with a minimum concentration equivalent to 0.0012 per cent arsenic pentoxide, although a concentration equivalent to 0.0015 per cent was necessary before sufficient injury occurred to be of importance. It was also apparent that slightly greater injury occurred when this quantity of soluble arsenic was applied in five sprays of 0.0003 per cent each than when it was applied in one spray of 0.0015 per cent arsenic pentoxide. This is probably due to the greater quantity of water furnished and to the smaller loss of poison in the former case. Plots

7 to 11 offer evidence that arsenic is a cumulative poison within the peach leaf, as sufficient rain intervened between applications to remove any arsenic left upon the surface of the leaf. In plot 7 sufficient arsenic remained within the leaves from two applications of 0.0003 per cent arsenic pentoxide to cause injury when a third application of 0.0009 per cent was made 13 days later, the injury being only slightly less severe than from one application of 0.0015 per cent. These results indicate that the disposal of this poison by the leaf is at least very slow and that arsenic may therefore be termed a cumulative poison.

SOLUBLE ARSENIC IN ACID LEAD ARSENATES

A summary is given in Table 4 of the toxic concentrations of arsenic acid and the equivalent percentages of soluble arsenic in powdered lead arsenate when used in the proportion of 1 pound to 50 gallons of water.

TABLE 4.—*Minimum concentrations of arsenic acid causing various types of injury to peach foliage*

Extent of injury	Minimum concentrations of soluble arsenic pentoxide causing injury specified	Equivalent to the percentage found in the spray when the lead arsenate (used in the proportion of 1 pound to 50 gallons of water) has a soluble arsenic content of—
	Per cent	*Per cent*
Trace of leaf burning	0.0012	0.50
Light leaf burning	.0015	.62
Moderate leaf burning	.0025	1.04
Severe leaf burning	.0050	2.08
Light defoliation	.0075	3.11
Complete defoliation	.0300	12.45
All twigs killed	.0500	20.75

TABLE 5.—*Soluble arsenic determinations in distilled water and in well water*

Sample No.	Soluble As_2O_5 present when digested at 32° C. for 24 hours in—		
	Distilled water		Well water used in spraying (pH 6.4)
	Unboiled (pH 5.7)	Boiled (pH 6.1)	
	Per cent	*Per cent*	*Per cent*
1	0.092	0.112	0.505
2	.172	.221	.650

If it be assumed that all the injury from the use of acid lead arsenate is caused by the arsenic that becomes soluble in the spray tank, it would appear from Table 4 that a lead arsenate with soluble arsenic

in quantities less than 0.50 per cent as arsenic pentoxide would be perfectly safe upon peach foliage. This of course refers to the soluble arsenic formed in the water used in spraying. According to the official methods (*1*), the solubility is determined in boiled distilled water which gives much lower values, as shown in Table 5, owing to its greater acidity and lack of buffering power. Where more alkaline waters are used in making up the spray, even greater differences result. Granting the above assumption, therefore, a lead arsenate containing less than 0.11 per cent of soluble arsenic pentoxide according to the official methods would seem necessary to prevent injury.

This assumption, however, is untrue, as arsenic has been shown to be a cumulative poison, and the repeated absorption of much smaller quantities of soluble arsenic by the leaves resulted in injury. Therefore, if water from frequent rains or dew is available upon the leaf for the hydrolysis of the lead arsenate, injury would be expected to occur even when an arsenate of very low soluble-arsenic content was used. The only safe acid lead arsenate would be one which was not at all hydrolyzed by water. This apparently is impossible to obtain, as even the purest acid lead arsenate is slowly hydrolyzed by the action of water according to the following equation (*7, p. 1918*):

$$5PbHAsO_4 + HOH \rightleftarrows Pb_4(PbOH)(AsO_4)_3 + 2H_3AsO_4$$

As the soluble arsenic acid thus formed is constantly being removed by passage through the leaf epidermis, the reaction continues to move to the right whenever moisture is available, and a concentration of arsenic sufficient to cause very severe burning or even defoliation often accumulates within the leaves.

Although it is apparently impossible to produce an acid lead arsenate with soluble-arsenic content so low that it will not burn the leaves, it is evident that one containing a high percentage of soluble arsenic is very dangerous to foliage.

FIELD-SPRAYING TESTS

To determine from a practical standpoint just how low the soluble arsenic should be for minimum injury, a series of lead arsenates with contents of soluble arsenic pentoxide ranging from 0.04 to 1.19 per cent were used upon peach trees during the summers of 1927 and 1928. These were applied with a double-acting hand pump at 160 pounds pressure. All were used in the proportion of 1 pound to 50 gallons of water and each tree was sprayed until the spray dripped from the leaves. All arsenates were analyzed by the official methods (*1*). These analyses and the results of the spraying tests are given in Table 6. High humidity and frequent rains throughout both of the experimental periods provided an abundance of moisture for the hydrolysis of the acid lead arsenate, conditions during 1928 being much the more favorable to burning in this respect.

DISCUSSION OF RESULTS

Perhaps the most important fact brought out by these experiments is that acid lead arsenate when used alone in the proportion of 1 pound to 50 gallons caused very severe injury even when the

soluble arsenic was as low as 0.04 per cent as arsenic pentoxide. Apparently the soluble arsenic can not be sufficiently reduced to prevent burning.

TABLE 6.—*Summary of field experiments in spraying peach trees with lead arsenate solutions (1 pound to 50 gallons) containing varying percentages of soluble arsenic pentoxide, performed during 1927 and 1928*

EXPERIMENTS OF 1927

Sample	Total As_2O_5	Total As_2O_5 (as As_2O_5)	Water-soluble arsenic as As_2O_5	Interval between application and—					Final extent of injury
				Initial injury	Moderate leaf burning	Initial defoliation	Severe defoliation	Complete defoliation	
	Per cent	*Per cent*	*Per cent*	*Days*	*Days*	*Days*	*Days*	*Days*	
1	31.29	0.16	0.13	4	7	6	14	----	Severe defoliation.
2	29.89	.31	.17	4	7	5	14	----	Do.
3	32.26	.45	.40	4	7	5	8	----	Very severe defoliation.
4	32.21	.45	.63	4	7	5	8	----	Do.
5	31.28	1.14	.88	3	7	5	8	8	Complete defoliation.
6	31.81	1.40	1.19	2	5	5	7	8	Do.

EXPERIMENTS OF 1928

A	32.80	.25	.04	4	7	11	22	31	Complete defoliation, very light twig cankering.
B	29.90	.40	.11	5	7	11	27	37	Do.
C	32.46	.44	.12	4	7	11	18	27	Complete defoliation, light twig cankering.
D	31.68	.44	.16	4	7	8	18	27	Do.
E	29.50	.30	.22	4	6	8	18	27	Do.
F	32.21	.45	.61	3	5	8	11	13	Complete defoliation, severe twig cankering.
G	31.28	.45	.82	3	4	6	11	13	Complete defoliation, very severe twig cankering.

There was no appreciable difference in the injury produced by a lead arsenate containing 0.04 per cent and by those containing up to 0.22 per cent water-soluble arsenic pentoxide. When arsenates containing higher percentages of soluble arsenic were used, the injury took place more rapidly and the final injury was more severe.

WATER-SOLUBLE ARSENIC IN CHEMICALLY PURE AND IN COMMERCIAL ACID LEAL ARSENATES

Chemically pure acid lead arsenate was prepared by twice crystallizing from nitric acid according to Duvillier's method (*8*). This was washed thoroughly with acidified water (pH 4.0), dried at 110° C. to constant weight, and analyzed. It was found to contain 33.0 per cent arsenic pentoxide and 64.1 per cent lead oxide, thus proving it to be pure acid lead arsenate. After being digested with boiled distilled water (pH 6.4) for 24 hours at 32° according to the official methods (*1*), it was found that 0.04 per cent of its weight as arsenic pentoxide had dissolved in water.

Commercial acid lead arsenates are practically never as low as this in soluble arsenic, even after repeated washing. Numerous analyses were made to determine the causes of the comparatively large quantities of soluble arsenic, and typical examples of these results are given in Table 7. All analyses were made by official methods.

TABLE 7.—*Soluble arsenic found in various samples of acid lead arsenate before and after washing*

Sample No.	Total As_2O_5	Total As_2O_5	pH [a]	Alkaline material present	Soluble arsenic as As_2O_5	
					Initial	After washing [b]
	Per cent	*Per cent*			*Per cent*	*Per cent*
1	0.450	32.21	5.4	None	0.601	0.063
2	.396	29.50	6.4	Excess lead oxide	.158	.132
3	.450	31.28	7.8	Calcium	.759	.139
4	1.400	32.40	5.4	None	1.190	.200
5 [c]	.130	33.00	4.8	do	.040	.040

[a] 1 gm. powder to 10 c. c. distilled water; shaken, and pH determined after 10 minutes.
[b] Washed four times with water acidified with nitric acid (pH 4.0).
[c] Chemically pure lead arsenate prepared by the writer.

SOURCES OF SOLUBLE ARSENIC IN THE SAMPLES

The soluble arsenic in sample 1 (Table 7) was greatly reduced by washing. Since analyses failed to disclose the reason for the large quantity of soluble arsenic present, a sample was boiled for one hour in water made acid with nitric acid (pH 3.0), dried, and again analyzed. The soluble arsenic content was then found to be 0.10 per cent as arsenic pentoxide, indicating that in this case sufficient time for reaction during manufacture had not been given.

In sample 2 the soluble arsenic was not greatly reduced by washing, and the greater quantities were apparently due to more rapid hydrolysis. The speed of hydrolysis of acid lead arsenate increases with an increase in OH-ion concentration, and the pH value of this sample was found to be somewhat higher than that of pure lead arsenate. This is apparently due to the excess of lead oxide, which has a slightly alkaline reaction. A large excess of lead oxide is apparently to be avoided, although a slight excess is unavoidable under commercial conditions.

In sample 3 also the large quantity of soluble arsenic was due to more rapid hydrolysis caused by higher alkalinity. In this case calcium was found to be present, and upon inquiry it was found that hydrated lime had been added during manufacture to neutralize any free acids present. The addition of materials with an alkaline reaction should always be avoided where possible.

In sample 4 the large quantity of soluble arsenic was apparently due to the arsenic trioxide present. The arsenic trioxide used in manufacture had been incompletely oxidized, and a small quantity was present in a loosely bound form, probably as lead arsenite. The soluble-arsenic content has invariably been observed to be high when a large quantity of arsenic trioxide is present in lead arsenate.

In sample 5 the soluble arsenic was apparently formed by hydrolysis of pure acid lead arsenate. The quantity formed was low and was not changed by repeated washing with acidified water.

No samples were found in which the soluble arsenic was due to the use of an insufficient quantity of lead.

EFFECT OF LOW SOLUBILITY UPON TOXICITY TO INSECTS

As it has been shown that injury can be reduced, but not prevented, by the reduction in soluble arsenic pentoxide below 0.25 per cent,

and that it is possible to produce a lead arsenate as low as this or lower in soluble arsenic, there yet remains the question as to whether the toxicity to insects is lowered by this reduction in solubility.

The digestive juices within an insect's digestive tract which are available for reaction with lead arsenate have been found (*2, p. 139; 4; 5*) to have a slightly alkaline reaction (pH above 7.0). Tests conducted by the writer have shown that hydrolysis of lead arsenate takes place rapidly in solutions with pH values above 6.0. Under these conditions such small differences in initial soluble arsenic would be expected to have little or no influence upon toxicity.

Cook and McIndoo (*3, p. 42*), as the result of rather extensive toxicity tests upon various insects with a number of arsenates, came to the following conclusions: (1) No differences in mortality could be attributed to the usual differences in the water-soluble arsenic content found in insoluble arsenates; (2) toxicity seemed to be based upon the stability of a compound and upon how easily it could be broken down in the bodies of insects.

Apparently the initial soluble arsenic, within ordinary limits, has little or no effect upon toxicity, and it would seem safe to conclude that the soluble-arsenic content of acid lead arsenate could be reduced below 0.25 per cent as arsenic pentoxide without loss in toxicity to insects.

SUMMARY

At low concentrations of equivalent arsenic content, arsenious and arsenic acids are equally toxic to peach foliage. At higher concentrations arsenic acid is the more toxic.

Arsenic acts as a cumulative poison within peach leaves.

The minimum concentration of arsenic acid toxic to peach foliage contains the equivalent of 0.0012 per cent of arsenic pentoxide.

Acid lead arsenates containing less than 0.25 per cent of arsenic pentoxide in water-soluble form gave minimum foliage injury. Nothing of practical importance was gained by further reductions in soluble arsenic.

It is apparently impossible to reduce the soluble arsenic in acid lead arsenate sufficiently to prevent serious injury when used upon tender foliage. It is therefore evident that acid lead arsenate can not be safely used upon susceptible plants without the addition of some material to prevent burning.

The initial soluble arsenic, within ordinary limits, has little or no effect upon the toxicity of acid lead arsenate to insects.

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INFECTION OF FRUIT OF CITRUS BY PSEUDOMONAS CITRI [1]

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INTRODUCTION

The present studies were made for the most part on citrus fruits grown in an isolated greenhouse, especially constructed for citrus-canker investigations, and situated near Washington, D. C. This paper gives findings regarding the invasion of the fruit tissues by the canker organism (*Pseudomonas citri* Hasse), the rate of multiplication of the bacteria in these tissues, and the resultant growth reactions of the fruit. In interpreting results and drawing deductions therefrom one must bear in mind that the environmental conditions in the greenhouse differ in many respects from those in commercial citrus plantings.

METHOD OF INOCULATION

Most of the data from this investigation are of a quantitative nature, based on the number of visible lesions developing under a given set of conditions when a certain number of puncture wounds are inoculated. In routine puncturing of citrus leaves for such quantitative infection tests it has been found convenient to use a simple punch consisting of 10 or 20 pins or needles stuck through a cork. This method of quantitative testing with punctured grapefruit leaves was used in preference to poured agar plates because of the elimination of irregularities due to the growth on plates of contaminating organisms that have an inhibiting effect on *Pseudomonas citri*.

In the case of young fruit it was found that the puncturing instrument ruptured some of the oil glands. The exuding oil had a tendency to injure a portion of the adjacent tissue and to interfere with a normal infection reaction. A definite test of the matter was made by making a large number of punctures singly with a needle directly into the oil glands, and by making another group of punctures elsewhere on the same fruit and taking care to avoid the oil glands. Similar inoculum of *Pseudomonas citri* was applied to both groups, and the number of infections was noted after the lapse of sufficient time for their full development. (Fig. 1.) The results of such a test are given in Table 1.

It is evident that from the standpoint of careful experimental technic it is necessary to avoid making punctures into the oil glands of the friut. Puncturing of oil glands was avoided in the various experiments described in this paper by making punctures very carefully, one by one, under a magnifier.

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TABLE 1.—*Percentages of citrus cankers resulting on fruit from the inoculation of punctures into and between oil glands*

Fruit and variety	Diameter of fruit	Cankers developed at punctures—	
		Into oil glands	Between oil glands
	Mm.	*Per cent*	*Per cent*
Ponderosa lemon	35	0	80
Otaheite orange	25	6	32
Pineapple orange	40	0	8

The inoculum was prepared from fresh, vigorously growing *Pseudomonas citri* cultures usually on potato plugs. These were checked closely to guard against diminution of virulence. Each batch was made up so as to be of approximately the same concentration. The inoculum was regularly applied to the fruit on moist cotton or cloth

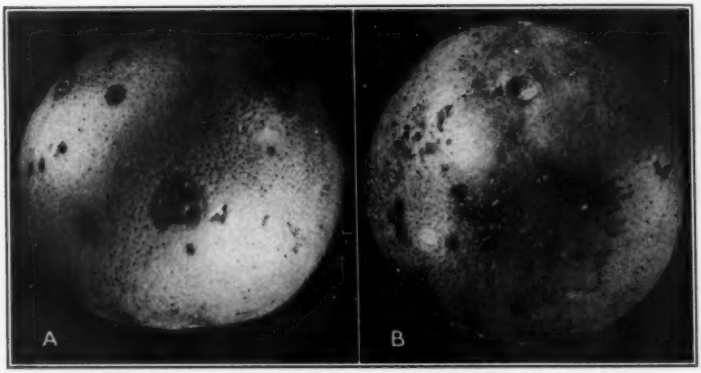

FIGURE 1.—Green grapefruit inoculated with a suspension of *Pseudomonas citri*. On one side (A) a group of punctures was made into the oil glands. On the other side (B) punctures were made between the oil glands. Extensive infection occurred in the latter instance, but none in the former. There are scattered stomatal infections where no punctures were made

swabs. These were left on under wrappings of several folds of paraffin paper, which were removed after a few days.

INFLUENCE OF AGE OF WOUND ON AMOUNT OF INFECTION

To determine what influence the age of the wound at the time of infection has on the percentage of canker lesions that will develop, several groups of 50 punctures between oil glands were made on susceptible young fruits at four different times, over a period of 50 hours. Until the end of the 50-hour period the fruits were left dry and unwrapped. At that time inoculum of the same concentration was applied simultaneously to all groups of punctures on all fruits. The results are given in Table 2.

Evidently wounds as much as 8 hours old when kept dry are not very likely to become infected. Only 3 out of 13 fruits developed any infection through wounds of this age, and such infection was only

one-tenth to one-fifth as great as developed through fresh wounds on the same fruits. These three and one other fruit are the only ones in this test that developed stomatal [2] infections at apparently uninjured places. The fruits with stomatal infections were the youngest ones in the test, as evidenced by their comparatively small sizes for their respective varieties. All of this might indicate a state of greater tissue susceptibility for these fruits; but other fruits showing equal or higher percentages of infection for freshly made wounds failed to develop any infection in the wounds that were 8 hours old.

TABLE 2.—*Percentages of green-fruit punctures which developed cankers after being kept dry for different periods before inoculation*

Fruit and variety	Diameter of fruit	Infection percentages, when the wounds dried for—				Number of stomatal infections per fruit
		Less than 1 hour	8 hours	26 hours	50 hours	
	Mm.					
Pineapple orange	46	20	0	0	0	0
Do	55	4	0	0	0	0
Ruby orange	38	8	0	0	0	0
Otaheite orange	20	20	2	0	0	3
Do	25	80	0	0	0	3
Do	36	12	0	0	0	0
Duncan grapefruit	42	20	4	0	0	1
Ponderosa lemon	21	44	6	0	0	5
Do	55	33	0	0	0	0
Key lime	30	48	0	0	0	0
Rangpur lime	40	50	0	0	0	0
Citron	34	2	0	0	0	0
Do	39	70	0	0	0	0

In another test the fruit was kept moist from the time of puncturing until inoculated. In this case infection occurred readily in wounds 24 hours old, but the percentages were lower than when the wounds were freshly made.

Perhaps here, as in the case of oil-gland punctures, the injury or killing of one or more layers of cells renders the substratum unfavorable for the development of infection. A fresh wound gives ready access to cells still living, but after the lapse of a few hours the progressive dying of exposed cells may present an obstacle to infection.

Under natural conditions the citrus-canker bacteria are disseminated mainly in water, and some of the greatest outbreaks of infection have occurred after violent storms that injured foliage and fruits. It might be inferred from these experimental tests that injuries such as thorn scratches, limb rubs, sand scurfs, or insect punctures or bites, occurring during dry weather, with the lapse of some hours before precipitation, would lead to comparatively little infection.

INFLUENCE OF STRENGTH OF INOCULUM ON AMOUNT OF INFECTION

Tests were made of the effect of using very strong as compared with weaker grades of inoculum on wounded citrus fruits selected so as to be as nearly as practicable of the same size. The strongest inoculum, indicated as I/1, was made by using one 3-day-old potato-cylinder

[2] For convenience the term "stomatal infections" has been used to indicate the infections arising at points not wounded, although it is recognized that some of these may have been in reality at unrecognized wounds.

culture of *Pseudomonas citri* in 200 c. c. of water. Dilutions of 1 in 20 and 1 in 400 were made from this. In Table 3 are given the results of one test carried out in this way. Others gave similar results.

TABLE 3.—*Percentages of wound infection of citrus fruits resulting from the use of various dilutions of inoculum*

Fruit and variety	Diameter of fruit	I/1 inoculum		I/20 inoculum		I/400 inoculum	
		Fruits	Average infection	Fruits	Average infection	Fruits	Average infection
	Mm.	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*
Otaheite orange	21–27	4	38	2	0.5	2	0.5
Parson Brown orange	34–37	1	25			1	2
Double-flowered orange	43	1	20			1	5
Chinese lemon	46	1	10	1	2		

The strongest inoculum produced the highest percentages of infection, and the resulting cankers appeared first and reached full size soonest. The same inoculum applied at the same time to punctured grapefruit leaves gave 100 per cent infection from the I/20 dilution and 38 per cent from the I/400 dilution. In other tests punctured fruits of grapefruit have only rarely exceeded 50 per cent infection even with inoculum as strong as the I/1 used in this test. This indicates that very much stronger inoculum, approaching 400 times as strong, is required to produce a given amount of infection on wounded fruits than on wounded leaves of grapefruit. Similar findings have been made for commercial oranges, lemons, and limes. In natural outbreaks of canker in orchards, leaves seem to be much more readily infected than fruits.

These points are further shown in a test with Otaheite orange leaves and fruits using strong and weak inoculum, prepared as indicated above, of I/1 and I/400 strengths. (Table 4.) In recording results four stages of development of a canker spot are recognized: (1) Watery stage, the first distinctly visible water-soaked appearance; (2) pimple stage, small definite spots very slightly, if at all, raised; (3) blister stage, spots distinctly raised and enlarged but not broken open; and (4) erumpent stage, corky development approaching fully formed cankers. Table 4 indicates by stages the rate of development of the canker lesions as well as the percentages of infection on Otaheite oranges. The development throughout was somewhat slow because of rather low night temperatures in the greenhouse in the winter, when this test was made.

With each strength of inoculum the canker development was greater and faster on mature leaves than on either size of fruit, and on the smaller and younger fruits than on the somewhat larger and older ones. The strong inoculum brought about a quicker development of canker than did the weak inoculum on leaves and on both sizes of fruits. Comparing effects of I/1 inoculum on fruits with those of I/400 inoculum on the mature leaf, it is seen that the increase of four hundredfold in strength of inoculum was not sufficient to cause infection on these green fruits comparable with that produced on the leaf by the weaker strength of inoculum.

TABLE 4.—*Percentages of infection and rate of development of cankers on punctured fruits and leaves of Otaheite orange, following the use of strong and weak inoculum*

Days after inoculation	Strong inoculum, I/1					
	Mature leaf		Small fruit (31 mm.)		Large fruit (44 mm.)	
	Per cent infected	Stage	Per cent infected	Stage	Per cent infected	Stage
2	0		0		0	
5	0		0		0	
7	20	Pimple	0		0	
9	60	Blister	0		0	
15	(a)		(a)		(a)	
19	100	Erumpent	(b)	Watery	(b)	Watery.
26	100	do	10	Erumpent	(b)	Do.
34	100	do	20	do	4	Pimple.
43	100	do	20	do	(c)	
50	100	do	40	do		

Days after inoculation	Weak inoculum, I/400					
	Mature leaf		Small fruit (34 mm.)		Large fruit (47 mm.)	
	Per cent infected	Stage	Per cent infected	Stage	Per cent infected	Stage
2	0		0		0	
5	0		0		0	
7	0		0		0	
9	15	Blister	0		0	
15	75	do	(b)	Watery	0	
19	80	do	(b)	do	0	
26	85	Erumpent	(b)	do	0	
34	85	do	(c)		(c)	
43	90	do				
50	90	do				

[a] No record made. [b] Few cankers. [c] Fruit dropped.

RATE OF MULTIPLICATION OF PSEUDOMONAS CITRI IN FRUIT LESIONS

The method used for estimating the relative numerical increase of bacteria in inoculated fruit punctures was as follows: Punctures were made in numerous groups of 10 each on the fruit to be tested. Inoculation was made with cotton swabs in the usual way, the inoculum being strong enough to insure infection. Later at various time intervals a given number of the groups of punctures, regularly 6 comprising a total of 60 punctures, were removed with a flamed disk cutter in such way as to include the full thickness of the peel. These 6 uniformly cut disks were thoroughly teased out in 20 c. c. of sterile water. A portion of the resulting bacterial suspension, designated as d/1, was further diluted 1 in 20, and the dilutions were continued in the same ratio as far as might seem requisite. These several dilutions were then used to inoculate freshly made punctures on grapefruit leaves, regularly using 200 punctures on each of five leaves on each of two plants, making 2,000 punctures for a test of each dilution of each sampling. Records were made in due time of the number of cankers developing on the test leaves. The senior writer

has previously estimated [3] by this method that 2 to 4 infections developing in 2,000 punctures give evidence of something like 30 viable organisms per cubic centimeter of inoculum. This ratio of approximately 10 organisms per cubic centimeter of inoculum to 1 infection per 2,000 punctures holds very regularly up to about 300 infections per 2,000 punctures. Table 5 gives the results of one such test.

TABLE 5.—*Increase of Pseudomonas citri in a 34-mm. Otaheite orange fruit, as evidenced by the number of infections per 1,000 punctures developing on grapefruit leaves from inoculum prepared from samples taken at stated times from the orange fruit*

Days after inoculation	Infections per 1,000 punctures developing from various dilutions of inoculum from samples from orange fruit				
	d/1	d/20	d/400	d/8,000	d/160,000
0	0	0	0		
2	80	6	0.5	0	
5	160	3	.5	0	0
7	1,000	975	55	8	0.5
9	1,000	500	14	0.5	0
15	1,000	1,000	1,000	100	28
19	1,000	1,000	1,000	75	11
26	1,000	1,000	1,000	57	8

Evidently there was a very definite and considerable increase during the first 15 days in the number of bacteria in the sampled fruit punctures. From the fifteenth to the twenty-sixth day there was no evidence of continued increase, but there was a suggestion of possible decline in numbers. Tests made on the fifth and ninth days seem to be erratically low. Comparing the showing of the second day with that of the seventh, and the latter with that of the fifteenth, and noting the dilution of the original inoculum required to produce about the same effect on the test leaves, it is seen that there was an increase of perhaps two to four hundredfold of bacteria in the fruit wounds during each of these two periods. This means about eight generations of bacteria, if a regular geometrical rate of increase is maintained, during a 5-day period in the first case and during an 8-day period in the second. With the probability of considerable slowing down or actual reaching of a standstill two or three days before the fifteenth day, one may presume that most or all of the increase for the second period was also during a 5-day period.

The Otaheite orange fruit used in this test was the 34-mm. small fruit receiving the weak I/400 inoculum as shown in Table 4. The test was run at a somewhat low range of greenhouse temperature during the winter. This fruit did not give external evidence of infection until the fifteenth day, when there was hardly more than an indefinite watery appearance, and it did not develop more decided symptoms before dropping after the twenty-sixth day.

The sampling taken on the fifteenth day in the d/160,000 dilution gave infection of 28 out of 1,000 or 56 out of 2,000 wounds on grapefruit leaves. This would indicate about ten times 56 or 560 organisms

[3] FULTON, H. R. DECLINE OF PSEUDOMONAS CITRI IN THE SOIL. Jour. Agr. Research 19: 207-223. 1920.

per cubic centimeter for the inoculum used. The estimate for the d/1 inoculum would, therefore, be 160,000 times 560 or 89,600,000 organisms per cubic centimeter. If this is regarded as an even 90,000,000, the 20 cubic centimeters of this inoculum would have an estimated bacterial content of 1,800,000,000. These were from 60 inoculated fruit punctures, only a few of which showed the first suggestion of canker development. This would indicate an average of about 30,000,000 organisms in each incipiently infected wound at the end of the 15-day period. Tests with samples from similar spots made immediately after inoculation gave negative results even in the d/1 dilution by amethod that shouldhave shown the presence of as many as 30 organisms per cubic centimeter of inoculum, corresponding to 10 organisms from each sampled puncture. This indicates an increase ratio of something like 1 to 3,000,000.

Tests parallel to those shown in Table 5 for the small Otaheite orange fruit were made for the other five inoculated fruits and leaves shown in Table 4, except that samplings were not begun until the ninth day for the strong-inoculum series, and additional samplings were made on the thirty-fourth and fiftieth days for parts that had not dropped previously.

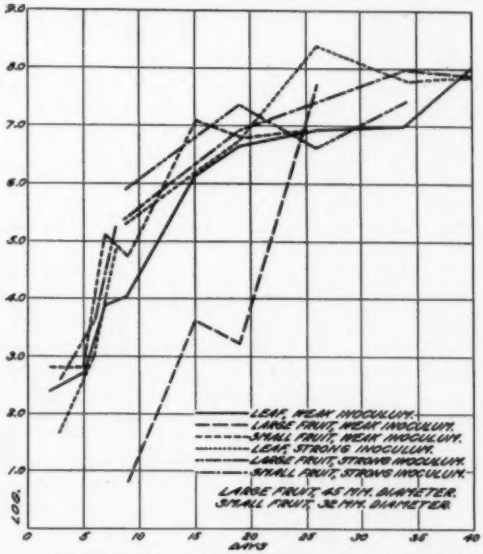

FIGURE 2.—Comparative rate of increase on a logarithmic basis of *Pseudomonas citri* in the tissue of large fruit, small fruit, and leaf of Otaheite orange following inoculation with strong (I/1) and weak (I/400) inocula. The breaks in the curves for the small fruit (strong inoculum) and the leaf (strong inoculum) indicate that the data were taken from two separate tests

In an earlier test made in September similar strong inoculum was used on a 34-mm. Otaheite orange fruit and on a mature leaf of the same plant, and three tests for bacterial increase were made on these at intervals during the first eight days. The data secured from this experiment seem to be concordant with the later results and are included in Figure 2.

The curves of Figure 2 were plotted to represent relative rates of increase of the bacteria in these six inoculated parts. The points on these curves were determined as follows: Relative estimates of the number of bacteria per infected puncture were made for each sampling by multiplying the dilution denominator by the number of infections produced per 2,000 leaf wounds; by multiplying this product by the factor 10 (average number of organisms per cubic centimeter of

inoculum to produce 1 infection per 2,000 leaf wounds), and by 20 (the number of cubic centimeters of inoculum); and by dividing the final product by 60 (the number of fruit punctures in the sample). An example of such a calculation is given on page 407.

To secure the greatest possible reliability for the estimates, two calculations were made for each sampling based on the results of two dilutions of inoculum selected as giving readings as nearly as possible within a range of 20 to 300 infection counts per 2,000 leaf punctures. The two estimates so obtained were averaged for the final estimate. The resulting number range from small to very large. To keep the graph within convenient size, the logarithms of the numbers were plotted rather than the numbers themselves. Since the multiplication of bacteria tends to be in geometrical progression, the plotting of logarithms gives a proper representation of such increase.

The indication from the increase curves of Figure 2 is that the bacteria multiplied with fairly equal rapidity in five of the six tests despite differences in host tissue and in strength of inoculum. The exception is the test of the larger fruit with the weak inoculum. Here there apparently was a slow start, followed by a very rapid increase to a maximum somewhat above that reached by four of the other five tests in the same 26-day period. The significant thing is that bacterial increase within the tissue should have been at so nearly the same rate while the development of visible lesions in the same material was so markedly different in both rate and extent of development, as has been shown in Table 4. For instance, on the ninth day for the strong inoculum neither of the fruits gave visible evidence of infection, while the leaf showed 60 per cent of the wounds at the blister stage. Inoculum prepared from samples of all three parts was of about equal potency on wounded grapefruit leaves. In the case of the three weak-inoculum tests there was an initial lag, which was overcome by the fifteenth day in two cases and by the twenty-sixth day in the third case. Generally speaking, approximately maximum development of bacteria was reached in about 15 days.

The test was repeated, using Otaheite orange of three sizes (40, 31, and 22 mm. in diameter) in comparison with mature leaves, and the same general results were obtained.

These experimental results raise a question as to what constitutes or measures susceptibility or immunity to citrus canker. Judging by external appearances as recorded in Table 4, one would say that the mature leaves of Otaheite orange are much more susceptible to wound infection than a 31-mm. fruit. But five samplings from the ninth to the fifty-sixth day gave indications of somewhat greater numbers of bacteria in the fruit than in the leaf for four of the five samplings. Even more striking is the comparison between the mature leaf and the small fruit receiving the weak inoculum. The development of an externally apparent canker is really a growth reaction of the host tissues. The presence of the causal organisms in sufficient numbers is a requisite for such development; but apparently under certain conditions the bacteria may be present in equal maximum number after having been equally active in multiplying within the tissue, and yet produce little or no canker reaction. In such cases the absence or weakness of canker development, usually taken to indicate resistance to the disease, does not seem to be due to any restraining

of bacterial activity, at least so far as their ability to grow and reproduce is concerned. This phenomenon constitutes what may be termed "quasi resistance" or "quasi immunity." This places emphasis on the importance of the reaction capacity of the host tissue rather than on the ability of the bacteria to flourish within the tissue as a determining factor in the production of canker lesions. The weak inoculum produced less visible effect than that four hundred times as strong (Table 4), but the results plotted in Figure 2 show that by the fifteenth day in two cases out of three the weak inoculations had reached approximately the same numerical level for bacterial invasion as the strong. While it might be assumed that a certain threshold number of bacteria must be present, or a certain quantity of bacterial metabolic activity must go on, in order to provide the proper stimulus for canker reaction in a given host-plant tissue, the present data indicate that the citrus tissue may be in a relatively nonresponsive condition without apparently offering any hindrance to *Pseudomonas citri* development, but in which the visible canker reaction is considerably limited or does not take place. Host tissues in different conditions of responsiveness may require different amounts of bacterial stimulus for a given effect, in which case the same amount of bacterial stimulus would be expected to produce different effects.

RATE OF MULTIPLICATION OF PSEUDOMONAS CITRI IN GREEN FRUITS OF VARIOUS CITRUS VARIETIES

Using the method already described, tests were made on green citrus fruits of various varieties and various sizes. These tests were made during the summer months, and the higher range of temperature gave a more rapid rate of multiplication of canker bacteria than that shown in Figure 2. The samplings were made at more frequent intervals. Conditions were favorable for blue-mold rot, and the fruits often became infected through the sampling wounds in spite of all precautions. Whenever blue-mold rot set in, tests for viable canker bacteria gave negative results. Even before the sampled portion actually rotted there was a considerable reduction in the number of bacteria. The effect of blue-mold rot on the persistence of the canker organism is considered further in another section of the paper.

Logarithms to the first decimal place have been calculated to represent the average rate of increase of bacteria per inoculated puncture, and the results are given in tabular form in Table 6 for three separate experiments.

The first samplings made after inoculation gave somewhat irregular results. If such sampling is made immediately after the inoculum is applied, the showing for viable bacteria is abnormally high because of inclusion of the residue of inoculum. If made after about an hour, when the fruit has become surface dry, the showing is many times lower or even negative. Since the rate of drying and consequent dying of superfluous bacteria is very variable, the results obtained for starting figures are irregular and sometimes abnormally high. Usually only one fruit of a series was sampled at the time of inoculation, and the estimate obtained was assumed to hold for all in the series.

TABLE 6.—*Relative logarithmic numbers of citrus-canker organisms estimated for infected punctures of green citrus fruits of various diameters and of leaves sampled at different times after inoculation, indicating the progressive numerical increase on a geometrical basis*

Experiment A				Experiment B							Experiment C			
Days after inoculation	Ponderosa lemon			Days after inoculation	Parson Brown orange		Pineapple orange		Satsuma orange (37 mm.)	Grapefruit leaf	Days after inoculation	Key lime (34 mm.)	Walters grapefruit (48 mm.)	Grapefruit leaf
	37 mm.	62 mm.	87 mm.		42 mm.	75 mm.	37 mm.	62 mm.						
0	3.3	3.3	3.3	0	2.0	2.0	2.0	2.0	2.0	2.0	0	0.6	0.6	0.6
1	6.1	5.6	6.2	1	5.6	4.8	5.6	3.9	5.7	2.6	1			
2			7.6	2	4.7	5.0	4.2	5.6	5.2	4.1	2	6.2	6.0	4.6
3	6.4	7.1	8.0	3	2.6	4.2	4.3	3.0	5.7		3			
4				4	2.8	1.8	5.2	2.6	4.9	4.2	4	(a)	(a)	4.6
5	5.4	5.9	6.4	6	(a)	(a)	5.5	(a)	5.5	4.9	5			
8	2.4	7.2	4.9	8			4.8		4.8	6.1	7			5.9
10	(a)	6.4	5.9	10			4.7			5.4	10			6.4
14		6.8	7.0	13					4.6	5.5				
19		6.5	1.9											
24		5.6	a 3.6											

a Fruit affected with blue-mold decay.

For the three Ponderosa lemons the rate of increase was much the same up to the fifth day, when the smallest one began to show a decline, the fruit being invaded by Penicillium. The 87-mm. fruit was similarly affected after the fourteenth day. This largest fruit did not develop any external evidence of canker reaction during the 24 days. The 62-mm. lemon developed 50 per cent of fully erumpent cankers during the test. The smallest (37 mm.) fruit showed 10 per cent of erumpent spots before its premature loss, and undoubtedly would have shown the greatest amount of canker reaction if it had persisted.

The two sizes of Parson Brown oranges and the two of Pineapple oranges showed definite increases to about the same general level. Unfortunately, three of these four fruits succumbed to blue-mold rot after the fourth day and before visible cankers could be expected. The remaining 37-mm. Pineapple orange developed 5 per cent erumpent cankers during the first 10 days. The 37-mm. Satsuma orange did not develop any external evidence of infection during the 19-day period. Field observations in canker-infested territory have shown the very high resistance amounting almost to immunity of Satsuma fruits to natural infection. In the present experiment the rate of increase in Satsuma fruit was fully as rapid as in the Pineapple orange of the same size, the latter developing normal symptoms of canker and the former none. The invasion through wounds and the subsequent multiplication of canker bacteria seem to have been closely parallel. One variety reacted in such a way as to form cankers, but the other variety did not visibly react. The grapefruit leaf in the same experiment gave lower tests in early stages than the orange fruits, but the maximum finally reached was approximately the same. The leaf by the thirteenth day had developed cankers of the blister stage at 100 per cent of the wounds.

The last section of Table 6 (experiment C) shows very rapid increase during two days in wounded fruits of key lime and Walters grapefruit, and the increase in wounded grapefruit leaf is given for comparison. Unfortunately, the two fruits were soon lost from blue-mold rot. The grapefruit leaves showed 90 per cent of visible canker in the blister stage on the tenth day. The possibility of wound infection without external reaction is thus demonstrated for a representative series of green citrus fruits. With increase in size of fruit beyond a certain point the development of external visible lesions is hindered, but the bacteria multiply practically as rapidly as when typical cankers are formed.

RATE OF MULTIPLICATION OF PSEUDOMONAS CITRI IN MATURE FRUITS OF VARIOUS CITRUS VARIETIES

Some of the inoculated fruits used in previous tests were approaching full size, but the peel was still green. It seemed desirable to test fruit in a more matured condition. Tests were made on fully colored fruit still attached to the trees in the greenhouse. The results are given in Table 7. A comparison with Table 6 shows very little difference in rate of increase between green and mature fruit. As mature fruit soon became infected with blue-mold rot, the tests ended prematurely.

TABLE 7.—*Relative logarithmic numbers of citrus-canker organisms estimated for each infected puncture of ripe citrus fruits sampled at different times after inoculation, indicating the progressive numerical increase on a geometrical basis*

Experiment A				Experiment B		
Days after inoculation	Pineapple orange	Duncan grapefruit	Ponderosa lemon	Days after inoculation	Pineapple orange	Ponderosa lemon
0	2.8	2.8	2.8	0	1.3	0.9
1	7.9	6.0	7.7	1	4.2	3.9
2	6.8	7.4	6.6	2	5.2	6.2
3				3	5.0	7.3
5	5.3	2.5	5.4	6	2.3	4.0

Mature fruits of orange, grapefruit, and lemon from the market were tested at two different times. In one instance the fruit was lost from decay after the fifth day; in the other it was held over a 28-day period and sampled every five days. In both tests the orange and the lemon gave negative results at every sampling after the first, which was made just after inoculation. The grapefruit in each case gave low estimates on the fifth day, indicating persistence with little if any multiplication. In the longer test the same condition was shown by the grapefruit on the tenth, fifteenth and twenty-first days, with negative results on the twenty-eighth day, the last testing. In still another experiment a mature grapefruit from the market was inoculated through wounds in the usual way, and 74 days later a test indicated something like 32,000 bacteria per puncture. This fruit had been held without intervening sampling and had not developed external lesions.

There is apparently a very marked difference in the behavior of the canker organism following inoculations in the peel of mature fruit after removal from the tree as compared with its behavior in the peel of mature fruit still on the tree. Possibly changes in the physiological condition of the fruit resulting from its removal from the tree are responsible for this difference. It is to be noted that in the cases of Diplodia and Phomopsis stem-end rots the advance of rot into the peel normally does not occur until some days or weeks after the fruit is removed from the trees, although there is abundant evidence that incipient infection already exists. Here the senescent changes in the peel favor the development of fungi having saprophytic tendencies; it is not inconsistent to presume that these changes would in equal degree hinder the development of an organism having definitely parasitic habits like *Pseudomonas citri*.

PERSISTENCE OF BACTERIA IN OLD CANKERS ON MATURE FRUITS

The question arises about the viability of *Pseudomonas citri* in typical canker lesions of mature fruits that originated while the fruit was young and green. In the greenhouse experiments certain fruits were inoculated while young and developed typical canker lesions. Six to seven months later, when these fruits were fully mature, tests were made on punctured grapefruit leaves in the usual way for evidence of viable canker bacteria. The test included two fruits of Pineapple orange, three fruits of Otaheite orange, two fruits of Pon-

derosa lemon, two fruits of Rangpur lime, one fruit of Temple orange, and one fruit of Duncan grapefruit. In every case the results were negative. (Fig. 3.)

From time to time fruits with well-developed cankers have been intercepted by quarantine inspectors and submitted for diagnosis. The obtaining of viable *Pseudomonas citri* from such specimens has been uncertain, although in a few instances successful cultures of the canker organism have been made from such material. (Fig. 4.) Infected fruits were too limited in number for a definite determination of the time limits of viability of the organisms in the lesions. This doubtless varies greatly with conditions.

EFFECT OF PENICILLIUM ROT ON VIABILITY OF PSEUDOMONAS CITRI

It was noted earlier in this paper that development of Penicillium rot in a fruit under test is regularly followed by a decrease in the number of viable canker bacteria recoverable. To test the matter definitely, mature orange fruits on the tree were puncture-inoculated at several locations with the canker organism. One day later *Penicillium digitatum* was introduced at one place on the fruit. Two days after this, when the softening from the blue-mold rot was apparent, tests were made from groups of punctures very near the advancing edge of the rot, but where the tissue had not yet softened, and also from punctured areas on the opposite side of the fruit. In one experiment the average of five tests from near the edge of the rotted area gave 5 infections per 1,000 grapefruit-leaf punctures for the d/1 inoculum, and a similar average for material from the opposite side of the fruit was 975 infections per 1,000 test punctures for a similar dilution of inoculum. In a second experiment samples taken from areas recently invaded by Penicillium gave negative results, while material taken at a distance gave an average of 403 infections per 1,000 test punctures.

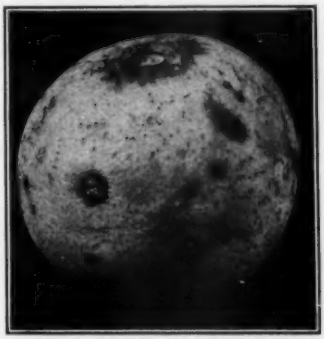

FIGURE 3.—Canker lesions on a mature Pineapple orange, the result of inoculation of the young fruit six months previously. These old cankers did not give evidence of containing viable *Pseudomonas citri* when culture and inoculation tests were made

INFLUENCE OF SIZE OF FRUIT ON DEVELOPMENT OF CANKER

In previous tests estimates were made quantitatively for the presence of the canker bacteria in the host tissues, and estimates of their numbers were made regardless of whether there were externally apparent lesions. Such testing requires much time and material. In ordinary practice reliance is put on the development of externally visible lesions to determine the fact and extent of infection. In the experiments that follow, this observational method was used to determine the range of fruit size or age in which infection is possible, either through wounds or without wounding, and the size of fruits giving the best development of lesions.

The method used may be illustrated by the following experiment: Four Homosassa oranges of approximately the same size, averaging 30 mm. in diameter, were punctured carefully between the oil glands, 50 punctures in each group, inoculated with standard inoculum on a swab, and wrapped. About 7 to 10 days later a new group of punctures was made in a different location on the same fruits, inoculum was applied, and the fruits were remeasured. The same procedure was followed until five successive tests had been made on these fruits, the last being when their average size was 50 mm. Close records were kept of the starting of visible infection at any group of wounds, or on the adjacent unwounded but inoculated surface.

In this test the average infection at the outset, at 30 mm. average size, was 47 per cent of erumpent lesions. The greatest average percentage of infections for any inoculation was 82 per cent at the 36 mm. average size, and these lesions reached a greater size than the others. At an average size of 48 mm. the lesions were still fairly numerous, averaging 41 per cent, but they were quite small, and none became erumpent. At 50 mm. average size there was only 9.5 per cent of infection, and the lesions were barely discernible as such. It thus appears, as judged by ordinary standards, that Homosassa oranges, under the conditions of this test, manifested greater susceptibility to canker infection through wounds at a size of about 36 mm. (nearly 1½ inches) in diameter than at smaller or larger sizes. The upper size limits of susceptibility to wound infection seemingly had been almost reached at the end of the test at an average size of 50 mm. (2 inches). In this test, fruits developing stomatal infections were more closely limited in size range, the smallest fruits showing any infection being the 29 mm. size and the largest 35 mm., with an average size of 32 mm. and an average of eight infections per fruit, all well developed. The beginning size for stomatal infections on this variety is not indicated by this test, and the optimum size is very likely smaller than the initial sizes used. Since inoculations were repeated three or four times after the fruits had passed the 35 mm. size, it may be safely concluded that this is about the upper limit of susceptibility for stomatal infection of this variety under the conditions of this experiment.

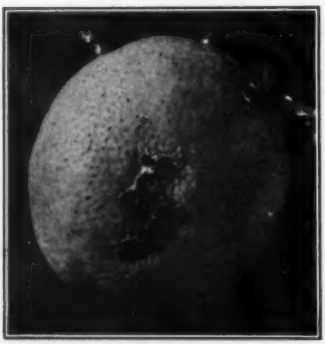

FIGURE 4.—Orange infected with canker, intercepted at San Francisco in the baggage of a passenger from Japan. Cultures or inoculation material from old lesions on such mature fruits may or may not give evidence of viability of *Pseudomonas citri*

In the same general way tests were made on a considerable number of varieties of green citrus fruits in the greenhouse. (Fig. 5.) Since the supply of such fruits was limited, it was not usually possible to select a group of the same size for inoculation at the same time. Inoculations were therefore made on fruits of any suitable size, proper measurements and records being made. It is recognized that size is by no means an exact indication of age, since individual fruits

may grow at different rates even when of the same variety, and the different varieties and classes of citrus fruits vary much in rate of growth and maximum size attained. This testing of varieties extended over three seasons, and naturally the various fruits used were subjected to a variety of growth conditions. An attempt was made to make inoculations as uniformly as possible, and with inoculum strong enough to produce a maximum of infection. Parallel inoculations were always made on punctured grapefruit leaves and always yielded practically 100 per cent of infection. Difficulty was experienced in getting records on fruits smaller than about 18 mm., because the injury caused by the puncturing and the covering of moist wrappings seemed to induce dropping. Records of infection were made after sufficient time had elapsed for infection to reach a

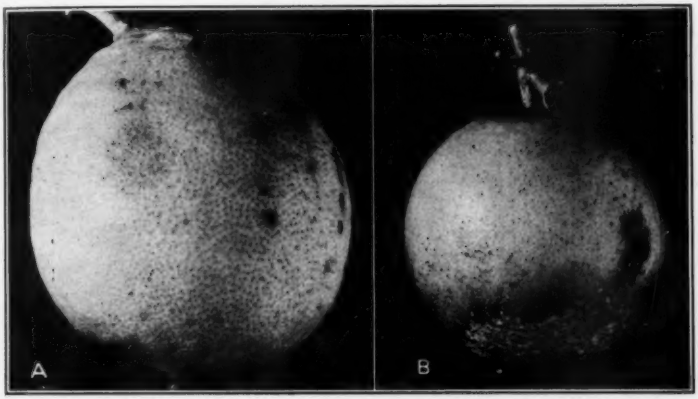

FIGURE 5.—A.—Ponderosa lemon. Five rows of 10 punctures each (partly showing) were made and inoculated when the fruit was 50 mm. in diameter. These developed 82 per cent infection. The circular group of 50 punctures at the left was made and inoculated when the fruit had reached a diameter of 65 mm., but no infection resulted. B.—Navelencia orange. The circular group of 50 punctures at the right side of the fruit was made and inoculated when the fruit was 28 mm. in diameter, and developed 30 per cent infection at wounds. There were also a few stomata lesions. Another group of 50 punctures (near the lower side of the fruit) was made and inoculated when the fruit had reached a diameter of 43 mm. These did not become infected

maximum, and distinction was made between wound and stomatal infections.

Table 8 gives the summarized results of these tests with particular reference to the relationship of fruit size to development of infection. In interpreting these results it must be borne in mind that the lower limits of infectable range could not be definitely determined because of the difficulty in getting very small sizes to persist after being inoculated. The poor condition of some that remained may have interfered with normal development of infection. If the fruits were not punctured, more of them remained attached, and so the test range could be carried lower for stomatal than for wound infections. It is noteworthy that the sizes that gave the greatest amounts of visible infection were usually at some distance from either the upper or the lower limits of the range tested, when any considerable number of tests were made for any variety. Toward the upper limits of infection the reaction lessened progressively from visible excres-

cences to mere external yellowing and finally to slight internal discoloration. It must be emphasized that the results in Tables 8 and 9 are not finally conclusive, and caution must be exercised in making any general deductions from them. In some instances the numbers of fruits tested were too low for reliable results. Numerical estimates based on the inoculated leaf puncture method of testing have shown the active invasion of fruits of Valencia orange to be about equal to Pineapple orange in one specific experiment, and that of Washington Navel to be distinctly less. In the same way invasion of fruit of Royal grapefruit was proved to take place, but in a less degree than in Marsh grapefruit. A single test to determine invasion of fruit of Nagami (oblong) kumquat gave negative results under rather unfavorable conditions for the test, due to the usually quick rotting of wounded kumquats.

TABLE 8.—*Results of inoculating fruits of various citrus varieties with Pseudomonas citri, showing the sizes most readily infected*

Fruit and variety	Data concerning wound infections					Data concerning stomatal infections				
	Tests	Size range tested	Infected fruits	Size range infected	Optimum size for infection	Tests	Size range tested	Infected fruits	Size range infected	Optimum size for infection
	Number	*Mm.*	*Number*	*Mm.*	*Mm.*	*Number*	*Mm.*	*Number*	*Mm.*	*Mm.*
Pineapple orange	54	15–58	36	19–58	40	60	8–58	10	22–43	31
Homosassa orange	30	17–54	23	17–54	36	36	17–54	3	29–35	30
Parson Brown orange	34	15–61	20	21–57	33	34	15–61	3	22–26	22
Mediterranean Sweet orange	11	21–49	4	21–48	25	15	16–49	1	21	21
Valencia (Lue) orange	9	25–49	0			9	25–49	0		
Navelencia orange	8	25–54	3	25–32	27	8	25–54	2	28–32	28
Ruby orange	4	38–58	1	38	38	4	38–58	0		
Washington Navel orange	3	18–49	0			3	18–49	0		
Satsuma orange	37	18–51	12	18–40	28	50	11–51	3	28–35	33
King orange	19	20–38	6	22–38	30	20	20–38	0		
Tangerine orange	17	16–42	2	23–28	28	22	11–42	1	24	24
Temple orange	4	38–60	0					0		
Otaheite orange	39	9–70	22	14–47	24	46	11–70	3	20–25	25
Double-flowered orange	14	25–48	6	33–46	42	17	19–48	0		
Myrtleleaf orange	13	16–45	3	25–36	30	13	16–45	0		
Willowleaf orange	3	30–40	1	30	30	3	30–40	0		
Duncan grapefruit	28	25–66	11	25–53	40	33	8–66	8	25–42	38
Walters grapefruit	11	28–65	3	28–48	48	13	15–65	2	40–48	40
Royal grapefruit	3	28–46	0			3	28–46	0		
Eureka lemon	9	30–45	3	33–36	33	11	21–45	0		
Lisbon lemon	10	21–40	1	38	38	9	21–40	0		
Kenedy lemon	10	23–38	2	27–35	31	11	17–38	0		
Villafranca lemon	6	27–45	1	27	27	7	27–45	0		
Lamb lemon	5	28–48	0			3	18–49	0		
Chinese lemon	19	30–53	8	30–46	36	19	38–53	1	35	35
Rough lemon	8	21–33	0			14	18–33	0		
Ponderosa lemon	45	17–92	25	21–88	35–75	54	11–92	9	21–70	56
Key lime	20	14–31	13	17–30	26	32	9–31	3	20–28	24
Rangpur lime	36	18–55	22	18–48	27	51	8–55	3	22–24	23
Sylhet lime	16	15–35	6	19–33	22	16	15–35	1	19	19
Suntara lime	8	18–32	6	18–29	23	8	18–32	1	21	21
Bearss lime	11	17–37	7	17–36	20	11	17–37	0		
Kusaie lime	18	11–32	3	18–32	20	22	7–32	0		
Dominican lime	18	14–36	3	16–26	21	21	9–36	0		
Woglum lime	2	34–36	0			2	34–36	0		
Tahiti lime	7	15–35	0			10	12–35	0		
Nagami kumquat	17	12–23	0			17	12–23	0		
Citron	9	34–44	3	34–39	39	9	34–44	0		
Citrus excelsa	5	12–28	1	18	18	6	12–28	0		

In Table 9 data from the same tests are used as a basis for quantitative expressions of infection for the various varieties. In the tabulation are included only those fruits that fall within the known infectible range of size for the particular variety. Not all fruits within such range actually developed infection in the tests. Several varieties in which neither wound nor stomatal infection was secured are omitted from this table. However, it is to be supposed that some of these might react under more suitable conditions or with more extended testing. Since the bases on which percentages of infection are calculated are reduced by this attempted elimination of sizes that may be immune, the resulting showing of infection is higher and supposedly a more correct index to the relative susceptibility of the various species. But caution must be exercised in drawing general conclusions from data having the unavoidable limitations of these. It must be recalled that what is here termed "susceptibility" is evidenced and measured by the outwardly visible reaction of the host tissues to the bacterial invasion. It has been shown in other cases that the bacterial invasion takes place just as truly, but the host tissues do not respond sufficiently to show any external effect. The present data do not take into account such invisible invasion.

TABLE 9.—*Results of inoculating infectible fruits of various citrus varieties with Pseudomonas citri*

Fruit and variety	Data concerning wound infections						Data concerning stomatal infections				
	Fruits of susceptible sizes			Infected fruits			Fruits of susceptible sizes			Infected fruits	
	Fruits	Infected		Punctures	Lesions	Punctures with lesions	Fruits	Infected		Lesions	Average lesions per fruit
	Number	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*
Pineapple orange	52	36	69	1,800	614	34	43	10	23	85	9
Homosassa orange	30	23	77	1,150	415	36	11	3	27	30	10
Parson Brown orange	34	20	59	1,000	176	18	12	3	25	31	10
Mediterranean Sweet orange	11	4	36	200	32	16	5	1	20	1	1
Navelencia orange	3	3	100	150	110	73	3	2	67	30	15
Ruby orange	1	1	100	50	4	8	------	0	------	------	------
Satsuma orange	35	12	34	600	99	17	49	3	6	27	9
King orange	19	6	32	300	27	9	------	0	------	------	------
Tangerine orange	10	2	20	100	6	6	6	1	17	10	10
Otaheite orange	29	22	76	1,100	221	20	12	3	25	10	3
Double-flowered orange	12	6	50	300	56	19	------	0	------	------	------
Myrtleleaf orange	10	3	30	150	9	6	------	0	------	------	------
Willowleaf orange	1	1	100	50	10	20	------	0	------	------	------
Duncan grapefruit	23	11	48	550	157	29	13	8	62	53	7
Walters grapefruit	7	3	43	150	45	30	6	2	33	25	13
Lisbon lemon	4	1	25	50	26	52	------	0	------	------	------
Eureka lemon	5	3	60	150	26	17	------	0	------	------	------
Kenedy lemon	8	2	25	100	64	64	------	0	------	------	------
Villafranca lemon	2	1	50	50	13	26	------	0	------	------	------
Chinese lemon	18	8	44	400	56	14	9	1	11	2	2
Ponderosa lemon	43	25	58	1,250	541	43	39	9	23	71	8
Key lime	20	13	65	650	116	18	20	3	15	13	4
Rangpur lime	32	22	69	1,100	425	39	11	3	27	9	3
Sylhet lime	16	6	38	300	81	27	3	1	33	5	5
Suntara lime	7	6	86	300	66	22	4	1	25	1	1
Bearss lime	11	7	64	350	52	15	------	0	------	------	------
Kusaie lime	17	3	18	150	105	70	------	0	------	------	------
Dominican lime	17	3	18	150	14	9	------	0	------	------	------
Citron	9	3	33	150	43	29	------	0	------	------	------
Citrus excelsa	1	1	100	50	3	6	------	0	------	------	------

INOCULATION OF FRUIT ON ORCHARD TREES

On account of the vigorous eradication campaign against citrus canker, it has not been possible for the writers to make test inoculations on orchard trees except in one instance. These few trees were outside of a commercial citrus region, in territory in which the eradication work was at the time suspended. The orange trees were seedlings, about 12 years old when the inoculations were made in May. There was already considerable old and fresh canker infection on the leaves of the lower limbs, but none was observed on the fruits. Fruits for inoculation were selected on the topmost branches above the drip from infected leaves. The fruits were punctured between the oil glands, 100 punctures each. Forty fruits were used, ranging in size from 18 to 38 mm., all but four being 25 mm. or larger. Strong inoculum was prepared by teasing well-developed leaf lesions in water until it looked turbid. The inoculum was applied on cotton swabs, and the fruit was wrapped with waxed paper. Punctured leaves were also inoculated on the same tree.

FIGURE 6.—Moist-chamber inoculation on detached green orange, 45 mm. in diameter

After one month, 16 fruits showed infection, all at wounds; 4 of them were in the 25-mm. class and averaged 4 infections per fruit, and 5 were in the 32-mm. class and averaged 3 infections per fruit. The greatest number of infections per fruit was 14 on a fruit of the 32-mm. class; the next was 12 on a fruit of the 38-mm. class; and the next was 6 on a fruit of the 25-mm. class. Of the 16 infected fruits, 7 had 1 infection per fruit. There was no increase in infection during the following three months. No stomatal infection developed on the inoculated fruits or on other fruits on the tree that had a good chance to receive washings from infected leaves. The artificially inoculated leaves developed 100 per cent infection.

While this test was too small in scope for any definite conclusions to be drawn, the behavior of the fruits growing naturally on large trees in this one instance was in general agreement with the experiments in the greenhouse in which green fruits on the trees were inoculated in a similar way.

INOCULATION OF DETACHED GREEN FRUITS IN MOIST CHAMBERS

Since the numbers and varieties of green citrus fruits available for inoculation in the greenhouse were quite limited, attempts were made to broaden the range by using young fruits that had been removed from the trees in Florida and sent to Washington. (Fig. 6.) These were punctured in the usual way, inoculated by dipping in a suspension of *Pseudomonas citri*, and placed in covered dishes lined with moist filter paper, care being taken to avoid too much

moisture. Small fruits, in general less than 20 mm. in diameter, were apt to decay quickly. The larger ones developed canker lesions rather more readily than did those on trees in the greenhouse, but the infection was much less abundant than was induced on leaves of the same variety with the same inoculum. Infection was secured on larger sizes of fruit in the moist chambers than on the trees. There was a tendency for a small proportion of uninoculated fruits, especially very small ones, to form spongy intumescences at the wounds that resembled early stages of canker, but did not develop into typical cankers. Microscopic examination of these showed the absence of bacterial ooze, and platings from them gave negative results for the presence of the canker organism. The inoculated fruits developed similar intumescences in greater numbers, and these always gave abundant evidence of bacterial invasion, both by microscopic examination and by plating, and the cankers in time developed to typical stages.

In the moist chambers there was the same tendency for the larger fruits to develop very slight external cankers, even though the bacteria had multiplied considerably in the tissues. This was determined by microscopic examination, by plating, and by inoculation of punctured grapefruit leaves with dilutions from the suspected lesions.

In one experiment fruits of several varieties and of uniform size for each variety were used in the moist chambers with three grades of inoculum indicated as I/1, I/20, and I/400. Some of the fruits receiving the I/1 inoculum were held in a refrigerator at about 45° F. The results are shown in Table 10.

TABLE 10.—*Percentages of infection developing on wounded citrus fruits kept in moist chambers after inoculation with Pseudomonas citri, using different dilutions of inoculum*

Days after inoculation	Fruits kept at room temperature (70° to 75° F.) after inoculation with dilution of inoculum indicated																		Fruits kept in refrigerator (45° F.) after inoculation with I/1 inoculum					
	Marsh grapefruit, 55 mm. diameter			Royal grapefruit, 53 mm. diameter			Pineapple orange, 45 mm. diameter			Washington Navel orange, 46 mm. diameter			Rangpur lime, 33 mm. diameter			Meyer lemon, 38 mm. diameter			Marsh grapefruit	Royal grapefruit	Pineapple orange	Washington navel orange	Rangpur lime	Meyer lemon
	I/1	I/20	I/400	I/1	I/20	I/400	I/1	I/20	I/400	I/1	I/20	I/400	I/1	I/20	I/400	I/1	I/20	I/400						
	P. ct.	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*		*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*
7	70	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	80	30	20	10	2	0	15	0	4	2	0	0	20	10	0	5	0	0	0	0	0	0	0	0
15	85	30	20	15	2	0	15	0	10	2	0	0	30	20	0	15	0	0	0	0	0	0	0	0
21	90	40	30	15	2	0	15	2	10	2	0	5	30	20	0	15	0	0	0	0	0	0	0	0
26	90	40	30	15	2	0	15	2	10	2	0	5	30	20	0	15	0	0	20	0	0	0	10	0
33	90	40	30	20	4	0	15	2	10	2	0	5	30	20	0	15	0	0	35	0	0	0	15	0
39	(a)	40	(a)	20	5	2	30	2	(a)	(a)	0	)	50	20	2	20	0	0	50	0	0	0	15	0
52	----	(a)	----	20	5	2	(a)	(a)	----	----	(a)	----	(a)	(a)	2	(a)	0	0	50	0	0	0	15	0
61	----	----	----	(a)	5	2	----	----	----	----	----	----	----	----	----	----	(a)	----	(a)	0	0	0	----	0
80	----	----	----	----	5	2	----	----	----	----	----	----	----	----	----	----	----	----	----	0	0	0	20	10
98	----	----	----	----	(a)	2	----	----	----	----	----	----	----	----	----	----	----	----	----	2	(a)	----	20	10
120	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	5	----	----	20	10

(a) Decayed.

Where strong inoculum was used the infection was evident sooner and developed in a larger percentage of wounds than where weaker inoculum was used. Marsh grapefruit developed lesions more quickly and extensively for each grade of inoculum than did Royal grapefruit of approximately the same size; and Pineapple orange gave similar evidence of having greater susceptibility than Washington Navel orange. Marsh grapefruit was first in apparent susceptibility, and Rangpur lime was second at both room temperature and in the refrigerator. The final reaction developed with I/1 at low temperature was about equal to that developed with I/20 inoculum on the same kind of fruit at room temperature of about 70° to 75° F., but the time required to develop such reaction was much longer at the lower temperature. In other words, at the lower temperature a much stronger inoculum as well as a much longer time was required to produce a given result.

The relative development of canker lesions on wounded fruits of grapefruit and orange is shown in Table 11. This test was made in moist chambers at room temperature, and three dilutions of inoculum were used. Observations were made over a 51-day period. The averages given are for five fruits in each treated lot. In Table 11 the term "blister" is used for any definite infection that had not become erumpent, and so it may have included the pimple or even the watery stage.

TABLE 11.—*Percentages of canker lesions developing on wounded green grapefruits and oranges held in moist chambers after being inoculated with three dilutions of inoculum*

Days after inoculation	Lesions produced on grapefruits (80 mm. average diameter) with dilution of inoculum indicated						Lesions produced on oranges (55 mm. average diameter) with dilution of inoculum indicated					
	I/1		I/20		I/400		I/1		I/20		I/400	
	Erumpent	Blister	Erumpent	Blister	Erumpent	Blister	Erumpent	Blister	Erumpent	Blister	Erumpent	Blister
	P. ct.	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*
14	14	26	1	14	0	0	1	1	0	0	0	0
20	32	19	4	15	0	2	3	7	0	0.6	0	0.4
28	46	19	18	27	6	8	6	21	0	6	0	1
38	47	25	28	30	8	14	12	20	1	11	0	3
51	47	27	28	30	9	15	12	26	1	18	0	3

The fruits were rather large, having been removed from the trees in August. The total amount of reaction in this moist-chamber test is much greater than developed in greenhouse tests of fruits of comparable size. The usual decrease in reaction with dilution of inoculum is noted. Oranges reacted much less in both amount and degree than did grapefruits. To produce a given effect on these oranges required an inoculum more than twenty times as strong (perhaps fifty to one hundred times as strong) as was required for the grapefruits. This is in keeping with the general results shown in Table 10 for smaller fruits.

In other moist-chamber tests Royal grapefruit developed canker less readily than Marsh, Duncan, and seedling grapefruit or shaddock, and Washington Navel oranges developed canker less readily than

Pineapple, Parson Brown, or Valencia. Fruit of Satsuma orange developed slight infection, even under most severe conditions. The results with King and Mandarin oranges were indefinite because of the early rotting of the fruit. Genoa lemon was on a par with Meyer lemon, and Villafranca and Kenedy reacted even less readily. Key lime fruits developed canker readily. Calamondin and kumquat decayed too quickly to give results.

The moist-chamber tests with wounded fruits were started in April and continued until August, successive lots of fruit being sent two or three times a month. The very smallest fruits used decayed too rapidly for canker to develop. Sizes of 12 to 25 mm. could be held long enough to show at least the beginnings of canker lesions. No infections were noted on fruits of such size range for any of the fruits tested. The smallest sizes to show infection in the various tests were as follows: Seedling grapefruit, 50 mm.; Marsh grapefruit, 54 mm.; Royal grapefruit, 53 mm.; Duncan grapefruit, 50 mm.; shaddock, 80 mm.; Pineapple orange, 40 mm.; Parson Brown orange, 41 mm.; Valencia orange, 38 mm.; seedling orange, 28 mm.; Satsuma orange, 35 mm.; key lime, 30 mm.; Buena Vista lime, 30 mm.; Rangpur lime, 33 mm.; Villafranca lemon, 38 mm.; Genoa lemon, 36 mm.; Kenedy lemon, 38 mm.; Meyer lemon, 38 mm.; Sampson tangelo, 35 mm.; King and Mandarin oranges and calamondin decayed too rapidly for infection to occur.

Not all of the above-named varieties were tested in every series, but those most important commercially were tested certainly once and often twice a month. The inoculum was regularly tested on wounded grapefruit leaves and was always potent enough for 100 per cent infection on these. There was certainly abundant opportunity for smaller sizes to become infected. Stomatal infections were very rare.

Mention has already been made of the practical failure to secure a canker reaction in fully matured fruits of orange, lemon, and grapefruit from the market. The large green fruits of orange and grapefruit in August gave strong reactions in moist chambers. On the whole, reaction was stronger for larger sizes of green fruit in the moist chambers than for similar sizes on the trees, and reaction started at larger sizes in the former case than in the latter. No ready explanation of this shifting suggests itself.

In the moist-chamber tests there was abundant multiplication of bacteria in tissue that did not react visibly, as was shown by using graded inoculum on punctured grapefruit leaves. But extensive tests to determine the rate of such increase and its limits as to size of inoculated fruits were not undertaken in the moist-chamber tests. Often a section cut through the inner peel showed a watery infiltration and a slight change in color, suggesting invasion by the bacteria, which microscopic examination confirmed, even when there was no external reaction.

It has been noted that a larger percentage of infections regularly develop in leaf wounds than in fruit wounds, when the same inoculum is used. It might be supposed that the inoculum penetrates less readily to the interior of the deep fruit punctures than to the open leaf punctures. To test this possibility detached wounded fruits were punctured and immersed in the inoculum under an exhaust so as to

withdraw air and cause thorough penetration. These fruits did not develop in moist chambers more infection than those inoculated in the ordinary way, and in both cases the infection was much less than in leaves inoculated at the same time with the same inoculum.

SUMMARY

A quantitative method is described for estimating from wound inoculations the number of *Pseudomonas citri* present at various stages in the development of canker lesions.

When punctures are made into the oil glands of citrus fruits, infection by *Pseudomonas citri* is seriously hindered. For dependable infection results the oil glands must be avoided in wounding for inoculation tests.

Fruit wounds as much as 8 hours old become infected much less readily than freshly made wounds. If allowed to dry the wounds decrease in infectibility more rapidly than if kept moist.

Weak grades of inoculum produce lower percentages of infection than strong grades, and the resulting cankers begin to show after a longer interval and develop more slowly. With a given strength of inoculum, infection is greater in amount and in degree on wounded leaves than on wounded fruits of a given citrus species.

The size of the fruit influences the amount and degree of canker development. It is difficult to secure wound infections on very small fruits, partly, perhaps, because of the damaging effect of the wounding and subsequent inoculation treatment. Intermediate sizes, from perhaps 25 to 35 mm. in diameter, show best development of canker lesions. Larger fruits develop lesions less readily. The exact upper and lower size limits of susceptibility vary with conditions and can not be inferred from the data at hand.

The majority of infections occur at visible wounds. The so-called stomatal infections occur most abundantly on somewhat smaller sizes of growing fruits than do wound infections, and stomatal infections fail to develop at an upper size range some 10 mm. less than for wound infections.

Periodical testing of infected wounds indicates that the multiplication of the canker bacteria takes place in about equal degree regardless of the size of fruit up to a stage of full maturity on the trees. Fully ripe fruit inoculated through wounds after removal from the tree did not give evidence of any definite increase of the canker organism. A practically maximum number of bacteria is reached in a few days at ordinary temperatures, after which a rather uniform level is maintained for a considerable time. The multiplication of bacteria in the tissues is independent of the development of external evidences of canker. In many instances where there was no visible symptom of canker the bacteria had multiplied just as freely as where there was normal canker development. Such a condition may be referred to as quasi immunity or quasi resistance.

A maximum development of bacteria in lesions may result from weak as well as from strong inoculum, a slightly longer period being required in the former case.

Development of Penicillium rot has a decided inhibiting or killing effect on *Pseudomonas citri* in recently developed lesions in the fruit peel.

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Various types and varieties of citrus fruits show differences in the readiness with which either wound or stomatal infections occur. The significance of this in judging relative susceptibility is lessened by the fact that host-tissue reaction may be lacking even when bacterial invasion and development have been at a maximum. The reactive condition of the host tissues is the important factor in canker development.

Tests for persistence of viable canker organisms indicate that they may die out within a period of five or six months, but under certain conditions they may persist for possibly longer periods.

Inoculation tests of limited scope on oranges under orchard conditions indicate general agreement with the findings from experiments in the greenhouse.

Infection tests of green fruits removed from trees and held in moist chambers also confirmed in essentials the findings from the greenhouse experiments. The principal differences were that fruits of the larger sizes developed visible canker lesions rather more readily than did those in the greenhouse tests and that infectibility started at a higher range of size.

At about 45° F. green inoculated fruit in a moist chamber required much stronger inoculum and a much longer time to develop approximately the same visible reaction as similar fruit held at a room temperature of about 70° to 75° F.

THE WOOLLY-KNOT TYPE OF CROWN GALL[1]

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INTRODUCTION

This article is a report of experiments which furnish evidence that a bacterial organism, tentatively referred to as the apple strain of the crown-gall organism, is the cause of malformations known as the woolly-knot type of crown gall, on root-grafted apple trees.

REVIEW OF LITERATURE

In 1907 Smith and Townsend (*18*)[3] reported the isolation of an organism from galls on the Paris daisy (*Chrysanthemum frutescens*) which they named *Bacterium tumefaciens*. Later Smith, Brown, and Townsend (*17*) isolated an organism from malformations on apple roots and referred to it as the apple strain of *Bact. tumefaciens*. Experiments were performed by these workers in an attempt to determine whether this organism was the cause of the malformations on the apple from which it was isolated, but owing to the rather limited extent of these experiments on the apple and to the fact that in many instances the results of the inoculations were negative or the controls became infected, the results were by no means conclusive.

Hedgcock (*2*) performed numerous experiments dealing mostly with the field control of crown gall and hairy root of the apple tree. Although proof was lacking, he expressed the following opinion:

> The period when most apple root grafts become diseased with crown gall is apparently the time when the wounds in the union are being healed by the formation of callus. If the disease could be kept out during this period it is quite probable that little would occur on nursery stock except as it gained entrance later through wounds made in cultivation.

In his recommendations for control he stressed the importance of making "close-fitting root grafts, avoiding blunt ends of the root and scion in the union."

Keitt (*3*) reported experiments in which grafts were made from carefully disinfected stocks and scions and planted in sterilized soil. He stated that grafts which are not well matched "developed enlargements of the types found in practice." He concluded that the largest overgrowths were produced by misfits in which the lower tips of the scions failed to unite satisfactorily with the stocks, while well-fitted and entirely matched grafts developed little or no excess callus. He noted that the overgrowths occurred where there seemed to be an interference in the stem of the plant.

Melhus (*4*) stated that "mechanical injury to the root may also lead to the excessive development of fibrous roots from the callus

[1] Received for publication Mar. 29, 1929; issued September, 1929.
[2] Credit is gratefully extended to R. B. Piper for most of the cultural and inoculation work in these experiments.
[3] Reference is made by number (italic) to "Literature cited" p. 449.

formed in the wound. This condition is not infectious." However, he also reported: "On seedlings * * * most of the so-called hairy root is not infectious, the infectious hairy roots arising from crown galls," and "so-called hairy root develops on callous tissue in a wound, from scions, buds, on the union, due to environmental conditions and due to inherent stock tendencies;" and later (*8*) "95 per cent of cases of crown gall are not caused by infection."

Riker and Keitt (*12*) reported that, in an examination of over 175 trees rejected as crown-gall trees in the nursery, the crown-gall organism was not found. They also stated that malformations resembling certain types of crown gall and hairy root were found on apple trees from grafts made aseptically and grown in steamed soil. They concluded that "of the several working hypotheses which might be advanced to conform with these results, the most promising one appears to be that the malformations dealt with on the rejected nursery trees were not induced by the crown-gall organism." They reported that their experiments indicated that fresh callus on apple graft "is not ordinarily an open infection court for the crown-gall organism."

Waite and Siegler (*20*) in making recommendations for the control of crown gall in the apple nursery stated that their results were not "in accord with the hypothesis of Riker and Keitt." They concluded that "it is difficult to assume that these malformations are due to any agency other than a pathogenic organism, since they are so definitely prevented by germicides."

Riker and Muncie (*14*, *15*) reported: "The common knot, which has been frequently called gall, appears in most cases to be merely excess callus." They mentioned satisfactory control by mechanical methods.

A summary of an address by Keitt (*1*) on investigations made by himself and his colleagues stated: "We have two types [of malformations]: (1) Knots formed by true bacterial crown gall, and (2) wound overgrowths." He continued: "One of the striking characteristics of wound overgrowths is root growth on the galls which is seldom present on true bacterial galls." The report on the use of fungicides, including Semesan (an organic mercury compound) as a means of control, concluded: "There were two ways in which chemicals may minimize the growth of galls: By extending the life of the wrapper; by dipping grafts in a strong solution a certain amount of injury to the callus is caused and the tip of the too long scion may be cut off as with a knife."

Melhus (*5*), in reporting as chairman of the crown-gall committee, Crop Protective Institute, stated: "That we have in the past confused excess callus called hard gall, with true crown gall; and nonpathogenic hairy root with pathogenic hairy root, can not be doubted."

Muncie (*6*, *7*) reported on two types of hairy root on French apple seedlings. The type which he designated the "woolly-knot form, arising from a distinct gall," was considered to be infectious and to have been induced by artificial inoculation of healthy seedlings with *Bacterium tumefaciens*.

Muncie (*7*) also reported cultures from overgrowths on piece-root apple grafts. This work, as well as that performed simultaneously by Riker and Keitt (*13*), has been discussed by Siegler (*16*), who

reported the isolation of an organism, tentatively referred to as the apple strain of *Bacterium tumefaciens*, from the most prevalent type (woolly knots) of malformations found on root-grafted apple trees. The necessity of using the proper host in testing for the infectiousness and the identity of organisms isolated from malformations on apple trees was emphasized. The fact that the woolly-knot type of malformation was identical with the wound overgrowth of Riker and Keitt (*13*) and Muncie (*7*) was also noted, but emphasis was placed on the fact that the illustrations of the malformations that these investigators had produced by growing apple grafts in steamed soil did not conform to the type commonly known as woolly knot. Evidence supporting the conclusion that this organism is pathogenic on the aboveground parts of apple as well as on other hosts was produced.

Patel (*9*) reported on the isolation of 15 nonpathogenic strains of *Pseudomonas* [*Bacterium*] *tumefaciens* from grafted apple trees. Subsequently, Patel (*10*) reported that the nonpathogenic strains caused no injury on a number of hosts, including apple.

Riker et al. (*11*) recently reported on "The relation of certain bacteria to the development of roots." They referred to Muncie's work wherein he stated that the woolly-knot malformation is a manifestation of crown-gall infection. They did not note that Muncie's illustrations (*7*), which show malformations that he concluded were merely wound overgrowths of noninfectious nature, were typical of what Hedgcock (*2*) had classed as the woolly-knot type of hairy root or crown gall.

The terms "hairy root" and "crown gall" are frequently used interchangeably. It has been customary for pathologists and nurserymen to refer to the woolly-knot type of hairy root as crown gall and not as hairy root. This type of malformation is essentially a knot or gall more or less covered with roots and, as noted previously by Siegler (*16*), is the type of malformation Riker and Keitt (*12*) referred to when they reported on the cultural examination of 175 apple trees rejected at the nurseries because of "malformations at the union (supposedly crown gall)," and later, as stated above, referred to by these writers as "wound overgrowth of nonbacterial origin."

Riker et al. (*11*) in a recent publication stated that in their previous investigations—

> were isolated organisms which were similar to *Bact. tumefaciens* in certain routine diagnostic characters, but which failed to induce positive evidences of infection when inoculated through punctures into stems of tomato, tobacco, Pelargonium or apple.

Recently these organisms were inoculated on the underground parts of 1 or 2 year old grafted apple trees of the Wealthy variety, and the writers stated that "at the end of the growing season no typical crown gall or hairy root was observed." They found, however, certain cases where small enlargements or sparse root development occurred. In another series of experiments in which the same inoculum was used, they reported the occurrence of fleshy roots in the places of inoculation. These fleshy roots "occurred singly or in groups and usually arose from slight enlargements." A period of approximately six weeks after inoculation was allowed to intervene before the readings on this experiment were made. In another series of experiments, young shoots of apples were inoculated with one of these strains which

previously had failed to infect when introduced through punctures into stems of the apple and other hosts, and in this series of experiments enlargements and excessive root development occurred. These workers discussed the differences in relation to both physiology and pathogenicity of this organism with what they designate as typical *Bacterium tumefaciens* and concluded that it is probably of specific rank. In referring to this so-called root-stimulating organism, they questioned whether it was harmful or beneficial to the plant, and they noted a possibility that it might be adapted to use in plant culture to facilitate root development.

EXPERIMENTS

M. B. Waite, in 1920, informed the writer that he had secured considerable control of crown gall of apple as early as 1909 by disinfecting with a solution of formaldehyde the scions and stock previous to grafting. In 1921 these experiments of Waite were repeated, and the results of his investigations were confirmed. In 1923 an organic-mercury compound was used in connection with disinfecting the scions and stocks before grafting. Excellent control was secured. These facts apparently supported the conclusion that the woolly-knot type of malformation is pathogenic in its nature. This conclusion was further supported by the fact that the writer had observed that galls on grafts treated aseptically up to the time of planting were, as a rule, smaller than those on grafts not so treated. It appeared probable that the smaller size of the galls on grafts which were kept under aseptic conditions for a brief period at least was due to the fact that infections occurred relatively late on them.

In 1925 a bacterial organism was isolated from malformations identical with the woolly-knot type of hairy root or crown gall, as illustrated by Hedgcock (*2*), and occurring at the graft union of apple trees. Inoculations with this organism caused malformations on Paris daisy stems. Before the isolations were made, macerations from the tissues of malformations on grafted apple trees had been inoculated into the daisy and slight reactions noted.

In a number of preliminary experiments, this organism, which in lieu of a better designation will be referred to herein as the apple strain of *Bacterium tumefaciens*, was inoculated on the roots of apple. Because the control trees frequently produced enlargements similar to those on the inoculated trees, the technic of these preliminary experiments was not considered satisfactory.

In 1927 a series of inoculations was made on 1-year-old seedling French crab-apple stocks. In addition to the apple organism a strain obtained from a peach gall was used. Small horizontal slits, approximately one-fourth inch in width, were made on the roots by means of a scalpel. The details of this experiment are given in Table 1. It should be noted that inoculations with the peach strain resulted in a higher percentage of infection than those with the apple strain; the controls were almost free from infection.

TABLE 1.—*Inoculations of 1-year-old French crab-apple seedlings with strains of Bacterium tumefaciens in 1927*

Experiment No.	Source of inoculum	Seedlings inoculated and planted	Seedlings surviving	Stand	Slits and root tips inoculated on surviving seedlings	Results of inoculations	
						Slits and root tips doubtfully galled	Slits and root tips galled
		Number	*Number*	*Per cent*	*Number*	*Number*	*Number*	*Per cent*
58–A	Apple strain (455–9–A–9)	25	14	56	84	7	12	14
58–B	Apple strain (461–A–9)	25	18	72	108	5	14	13
58–C	Apple strain (474–B–1)	25	23	92	138	4	18	13
58–D	Apple strain (486–1)	25	24	96	144	3	7	5
58–E	Apple strain (487–6)	25	19	76	114	12	13	11
Total	Apple strains	125	98	78	588	31	64	11
58–F	Peach strain	25	17	68	102	9	30	29
58–G (control)	Sterile water	25	18	72	108	11	2	2
58–H (control)	Sterile knife	25	11	44	66	7	2	3
Total	None	50	29	58	174	18	4	2.3

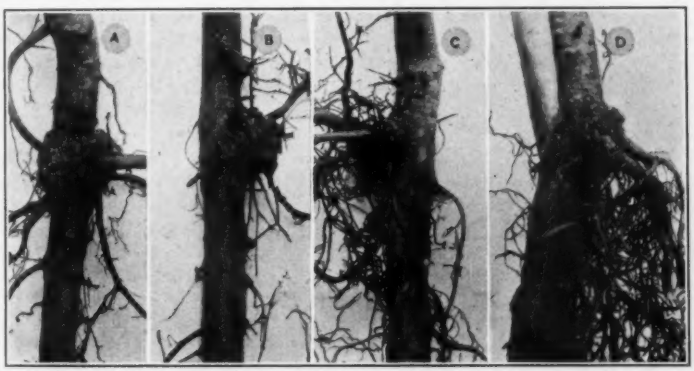

FIGURE 1.—Typical malformations produced on apple-seedling roots: A and B, Inoculated with the apple organism; C and D, inoculated with the peach organism. Only the apple organism was recovered from all four specimens. Slightly reduced

The type of malformation, however, was identical, regardless of whether the seedlings were inoculated with the apple or the peach strain or were uninoculated. It was, therefore, not surprising to isolate only the apple strain from typical specimens in each of these three lots. Figure 1, A and B, shows typical malformations obtained on the seedlings inoculated with the apple organism, and Figure 1, C and D, typical malformations obtained on the seedlings inoculated with the peach organism. The malformations on the controls were identical with these. Isolations were made from the galls illustrated in Figure 1 and from malformations on the controls, and the isolated organism, typical of the apple strain, was tested on various hosts and its identity proved.

The explanation offered to account for the high precentage of malformations obtained on the comparatively few seedlings inoculated with the peach organism is that natural infection occurred. This explanation is based on the facts that the apple strain, and not the original inoculum, the peach strain, was isolated from these galls, and that the galls were typical of those produced by infection with the apple strain and were not the smooth, rounded galls obtained by inoculation with the peach organism or with other strains obtained from similar apple galls. Figure 2 illustrates the type of gall obtained by an artificial inoculation with the peach organism which is probably identical with the daisy and the peach strains of Smith et al. (*17*). It is practically identical with the type of gall that Riker and Keitt (*13, pl. 38*) and Muncie (*7, pl. 2, A*) considered as the only pathogenic type. Further consideration of this subject will be found under "Discussion," but it should be noted that the explanation given above loses some of its plausibility when the fact that the controls were practically clean is taken into consideration. As the results of this experiment did not afford an interpretation that might be considered conclusive, they are given for information only, and not as confirmatory evidence of the pathogenicity of these two strains.

FIGURE 2.—Type of malformation on apple-seedling root resulting from inoculation with the peach strain. Natural size

In 1927 apple grafts were inoculated by being soaked in heavy suspensions of the organisms in sterile water. This experiment was undertaken to secure evidence concerning the pathogenicity of the apple and the peach organisms and the stage at which apple grafts are most susceptible to infection. The fact that a surface disinfection of scions and roots before grafting had given a considerable degree of control indicated that infection may occur quite generally before the grafts are fully callused. This point was mentioned as a probability by Hedgcock (*2*) and was supported by an experiment wherein grafts were disinfected at definite stages in their development. (Table 2.) The same disinfectant, a 0.25 per cent solution of hydroxymercurichlorophenol, has been used throughout these experiments. Disinfecting the scions and stock gave practically the same control as disinfecting the freshly made grafts, but disinfecting the grafts after they had callused resulted in a high percentage of galled trees.

TABLE 2.—*Results of disinfecting Jonathan apple grafts at different periods*

[100 grafts treated at each period]

Treatment	Resulting trees			
	Number dug	Number smooth	Number galled	Per cent galled
Scions and stocks disinfected before grafting	69	64	5	7
Grafts disinfected before callusing	68	61	7	10
Grafts disinfected after callusing	71	46	25	35
None	56	29	27	48

Accordingly, grafts aseptically made were immersed for definite periods in various inocula, either at the time they were made; that is, before callusing, or just previous to planting, after they had callused. The details of this experiment are given in Table 3. Although the percentage of infection obtained by inoculating the grafts at the time of grafting was extremely large as compared with that obtained by inoculating the grafts after they had callused, and although the control grafts and the grafts inoculated with the peach organism remained practically free of infection, the evidence was considered as of supplemental value only, because the numbers used were rather small and the stand obtained was poor. A definite indication, however, was given to strengthen the hypothesis that the freshly made graft was an open court for infection for at least one strain of the crown-gall organism.

In Table 3 are also included data obtained by making two horizontal slits on the scion part and two on the root part of each graft. In these cases the grafts were allowed to callus, the slits were made, and the grafts were then immersed in the inocula as indicated. The result of this part of the experiment was considered to furnish evidence concerning the pathogenicity of the apple strain. Only indirectly did it support the main thesis that the woolly-knot type of crown gall at the graft union is pathogenic in nature and is not merely a mechanical wound overgrowth. In contrast to the result obtained on apple seedlings reported in Table 1, the control slits and the slits inoculated with the peach strain remained free of infection. This supports the explanation of natural infection given to account for the results obtained in the previous experiment.

The experiments of 1927 were repeated with slight modifications in 1928. One-year-old apple seedlings as well as grafts of different varieties on various stocks were used. The seedling apple trees were originally obtained from seed of several open-pollinated varieties, and the grafts were made with stocks which were clons of seedlings obtained from several varieties, furnished by Guy E. Yerkes, of the Bureau of Plant Industry. This, of course, made for uniformity in the experiment. In the 1928 experiments the inoculated and control seedlings were allowed to callus for 22 days before they were planted, in order to keep the wounds free from contaminants after planting. The seedlings were inoculated by making small horizontal slits in them and then soaking them in suspensions of the inoculum. The results of these inoculations are given in Table 4.

TABLE 3.—*Inoculations of apple grafts with strains of bacterium tumefaciens in 1927*

Experiment No.	Host	Source of inoculum	Time of applying inoculum with reference to callusing	Grafts planted	Grafts surviving	Stand	Results of inoculation						Scion slits inoculated	Scion slits galled		Root slits inoculated	Root slits galled	
							At graft union			At root tip								
							Doubtfully galled	Galled		Doubtfully galled	Galled							
				Number	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*
38-A	Buckskin on French crab.	Apple strain (455-9-A-9)	Before	60	4	6.6	0	3	75.0	0	4	100						
38-B	do	Apple strain (461-A-9)	do	78	14	17.9	0	14	100	0	14	100						
38-C	do	Apple strain (474-B-1)	do	56	16	28.5	0	14	87.5	0	13	81.2						
Total	do	Apple strains	do	194	34	17.5	0	31	91.1	0	31	91.1						
38-D	do	Peach strain	do	65	26	40.0	1	0	0	1	0	0						
38-E (control)	do	Sterile water	do	45	33	73.3	0	0	0	0	3	9.1						
38-F	do	Apple strain (455-A-9)	After	45	23	52.2	1	1	4.3	2	10	43.5						
38-G	do	Apple strain (461-A-9)	do	45	26	57.7	1	4	15.4	0	10	38.5						
38-H	do	Apple strain (474-B-1)	do	45	33	73.3	1	3	9.1	2	8	24.2						
Total	do	Apple strains	do	135	82	60.7	3	11	13.4	4	28	34.1						
38-I	do	Peach strain	do	45	35	77.7	2	0	0	0	0	0						
57-A	Givens on French crab.	Apple strain (455-9-A-9)	do	35	18	51.4	0	4	22.2	0	2	11.1	36	9	25.0	36.1	13	36.1
57-B	do	Apple strain (461-A-9)	do	35	24	68.5	0	4	16.6	0	0	0	48	13	27.1	48	15	31.3
57-C	do	Apple strain (474-B-1)	do	35	19	54.2	0	3	15.7	0	0	0	38	4	10.5	38	11	28.9
57-D	do	Apple strain (486-1)	do	35	23	65.7	0	1	4.3	0	0	0	46	17	36.9	46	21	45.6
57-E	do	Apple strain (487-6)	do	35	27	77.1	0	1	3.7	0	0	0	54	13	24.1	54	22	40.7
Total	do	Apple strains	do	175	111	63.4	0	13	11.7	0	2	1.8	222	56	25.2	222	82	36.9
57-F	do	Peach strain	do	20	11	55	0	0	0	0	0	0	22	0	0	22	0	0
57-G (control)	do	Sterile water	do	20	13	65	0	0	0	0	0	0	26	0	0	26	0	0

TABLE 4.—*Inoculations of 1-year-old apple seedlings with the apple strain of Bacterium tumefaciens in 1928*

Experiment No.	Seedling host	Seedlings inoculated and planted	Seedlings surviving	Stand	Slits and root tips inoculated on surviving seedlings	Results of inoculation				
						Slits galled		Root tips doubtfully galled	Root tips galled	
		Number	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*
136-A	Delicious	54	53	98.1	159	56	52.8	2	1	1.8
136-A (control)	....do	54	49	90.7	147	0	0	2	0	0
136-B	McIntosh	50	45	90.0	135	40	44.4	4	13	28.8
136-B (control)	....do	48	38	79.1	114	0	0	4	0	0
136-C	Tolman	100	88	88.0	264	72	40.9	0	70	80.0
136-C (control)	....do	100	88	88.0	264	0	0	8	0	0
136-D	Northern Spy	54	45	83.3	135	50	55.5	6	14	31.1
136-D (control)	....do	50	43	86.0	129	0	0	4	0	0
136-E	Yellow Siberian	55	54	98.2	162	102	94.4	0	30	55.6
136-E (control)	....do	50	47	94.0	141	0	0	5	0	0
Total (inoculations)		313	285	91.1	855	320	56.1	12	128	44.9
Total (controls)		302	265	88.1	795	0	0	23	0	0

It is believed that the numbers used, the stand that was obtained, the uniformity of the stock, the high percentage of galls resulting on the inoculated trees, and the freedom of the controls from infection are all factors that should be considered as justifying the drawing of definite conclusions from the results of this experiment. In this experiment as well as in subsequent ones the seedlings were disinfected with an organic-mercury solution before they were used. The disinfecting solution was always washed off with distilled water. The fact that the seedlings in the controls were also disinfected and that the wounds were partially closed by callus before planting may account for their rather unusual freedom from natural infection. It is noted that of the 855 slits and root tips where the organism was afforded opportunity to infect, 448, or 52.4 per cent, became infected. On the 795 control slits and root tips no infection was found. The groups of inoculated and uninoculated Yellow Siberian seedlings are illustrated in Figures 3 and 4, respectively. Typical malformations resulting from inoculation with the apple organism and the controls free of galls are shown in Figure 5, A and B, and Figure 5, C and D, respectively. Reisolations from these galls, typical of those always resulting from infection with the apple organism, were not attempted in this case.

The series of experiments on apple grafts in 1928 gave results entirely in accord with those obtained on the apple seedlings in 1928. (Table 5.) The preponderance of infection resulting from subjecting the newly made graft to the inoculum previous to callus formation as compared with the infection of those grafts which were subjected to the inoculum after they were allowed to callus is evident. Again, as in the experiments of the preceding year the grafts inoculated with the peach organism and the control grafts remained free of galls. The total freedom of these control grafts from natural infection may be due to the disinfection of the scions and stock or to the absence of the inoculum in the soil in which the grafts were planted.

TABLE 5.—*Inoculations of apple grafts with strains of Bacterium tumefaciens in 1928*

Experiment No.	Host	Source of inoculum	Time of applying inoculum with reference to callusing	Grafts planted	Grafts surviving	Stand	Results of inoculation						Scion slits inoculated	Scion slits galled		Root slits inoculated	Root slits galled	
							At graft union			At root tip								
							Doubtfully galled	Galled		Doubtfully galled	Galled							
				Number	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*	*Number*	*Number*	*Per cent*
127-B	Northwestern Greening on French crab.	Apple strain (486-1)	After	94	70	74.5	4	3	4.3	1	0	0	140	42	30	140	63	45
127-C	do	Peach strain	do	94	63	67.0	2	0	0	2	0	0	126	0	0	126	0	0
127-A (control)	do	Sterile water	do	94	67	71.3	0	0	0	0	0	0	134	0	0	134	0	0
128-A	Yellow Transparent on Yellow Siberian.	Apple strain (486-1)	Before	50	28	56.0	0	26	92.9	3	3	10.7						
128-B	do	Peach strain	do	50	41	82.0	1	0	0	0	0	0						
128-D	do	Apple strain (486-1)	After	50	45	90.0	0	4	8.9	2	8	17.8						
128-E	do	Peach strain	do	50	38	76.0	4	0	0	0	0	0						
128-C (control)	do	Sterile water	Before	50	38	76.0	0	0	0	0	0	0						
129-A	Winter Banana on French crab.	Apple strain (486-1)	do	100	67	67.0	0	49	73.1	9	30	44.8						
129-B	do	do	do	100	73	73.0	8	27	37.0	2	13	17.8	146	1	0.7	146	5	3.5
129-E	do	do	After	100	72	72.0	2	3	4.2	1	0	0						
129-F	do	do	do	100	62	62.0	0	7	11.3	2	2	3.2	124	43	34.6	124	36	29
129-C (control)	do	Sterile water	Before	100	57	57.0	3	0	0	0	0	0						
130-B	Yellow Transparent on Florence crab.	Apple strain (486-1)	do	57	35	61.4	2	28	80.0	3	24	68.5						
130-C	do	Peach strain	do	57	38	66.7	5	0	0	0	0	0						
130-A (control)	do	Sterile water	do	57	41	71.9	5	0	0	0	0	0						
131-B	Delicious on French crab.	Apple strain (486-1)	do	75	21	28.0	1	17	81.0	0	9	42.8						
131-C	do	Peach strain	do	75	30	40.0	0	0	0	0	1	3.3						
131-A (control)	do	Sterile water	do	75	36	48.0	0	0	0	0	0	0						
132-B	Yellow Transparent on French crab.	Apple strain (486-1)	After	50	23	46.0	3	9	39.1	4	3	13.0						
132-A (control)	do	Sterile water	do	50	38	76.0	6	0	0	0	0	0						
Total (5 lots)		Apple strain (486-1)	Before	382	224	58.6	11	147	65.6	17	79	35.3						
Total (5 lots)		do	After	394	272	69.0	9	26	9.6	10	13	4.8						
Total (6 lots, control)		Sterile water		426	277	65.0	14	0	0	0	0	0						

Galls typical of those appearing on the grafts inoculated with the apple strain are shown in Figure 6, A–C. The organism was reisolated from the specimen in Figure 6, B, which showed unusual freedom from root developments on its surface. Attention is directed to the data given in Table 5 under the heading "Results of inoculation at graft union, galled" for the varieties Yellow Transparent on Yellow Siberian seedlings and Winter Banana on French crab seedlings, where

FIGURE 3.—Entire lot of 54 Yellow Siberian seedlings, showing numerous woolly-knot malformations resulting from inoculations with the apple organism in experiment No. 136–E. (Compare with Figure 4.) Greatly reduced

a comparison of grafts inoculated both before and after callusing may be found.

When attempts have been made to secure galled trees at certain periods of the year, especially in midsummer, there has always been difficulty in finding abundant specimens of galls on grafts planted the preceding spring. This fact led to the hypothesis that the high nutritional condition of the host at the point of infection may be a

factor of greater importance in the degree of pathogenicity exhibited than the state of rapid growth of the tissues at that point. The conception that rapidly growing tissues favor infection has been generally accepted. In order to test this hypothesis the following experiment was performed. Apple roots were inoculated by means of small horizontal slits, approximately one-fourth inch in width and one-eighth inch in depth, at two periods in midsummer. The inocu-

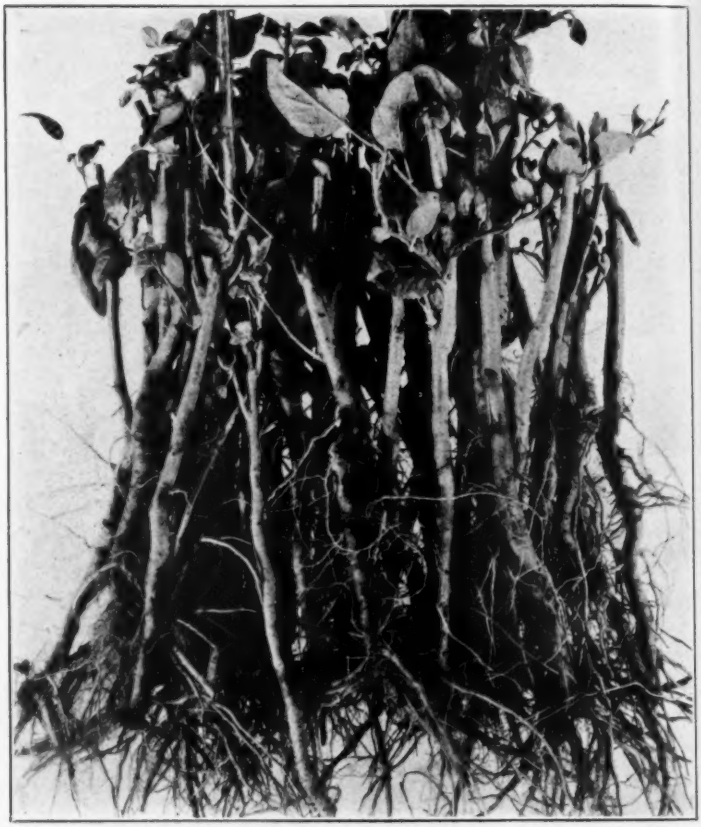

FIGURE 4.—Entire lot of 47 uninoculated Yellow Siberian seedlings, free of malformations. (Compare with Figure 3.) Greatly reduced

lations were made by inserting the inoculum from an agar slant of the apple organism into a slit made by means of a scalpel on the scion wood from one-fourth to 2 inches above the union, on grafts which had been planted the current spring. The details and results of these experiments are given in Table 6. Although the number of grafts used in this experiment was not large, it is felt that the results are so definite that, when coupled with the results of other experiments reported here, there is presented proof that the so-called apple strain

of crown gall is responsible for the type of woolly-knot galls found on grafted apple trees. It should be noted, however, that in this experiment inoculations were made on the scion wood and not on the graft union itself.

Galls resulting from inoculations made on August 20 were as large as those obtained from inoculations made on July 19. The roots on

FIGURE 5.—A and B, Woolly-knot galls on apple-seedling roots resulting from inoculations from the apple organism. C and D, Control seedlings, free of malformations. All natural size

the galls of trees inoculated on July 19 were mostly of the fibrous type, whereas the roots on the galls of the trees inoculated in August were invariably of the fleshy type. Typical illustrations of galls from these lots are shown in Figures 7, A and B, and 8, A to C, respectively. The organism was reisolated from the specimens illustrated in Figures 7, A and B, and 8, B and C.

TABLE 6.—*Results obtained by inoculating Yellow Transparent scion tissue adjacent to the graft union with the apple strain of Bacterium tumefaciens on July 19 and August 20, 1928*

Experiment No.	Date of inoculation	Grafts inoculated	Grafts surviving	Slits inoculated on grafts	Slits galled	
		Number	*Number*	*Number*	*Number*	*Per cent*
163	July 19	15	13	13	13	100
163 (control)	do	15	14	14	0	0
175	Aug. 20	50	50	50	50	100
175 (control)	do	25	25	25	0	0

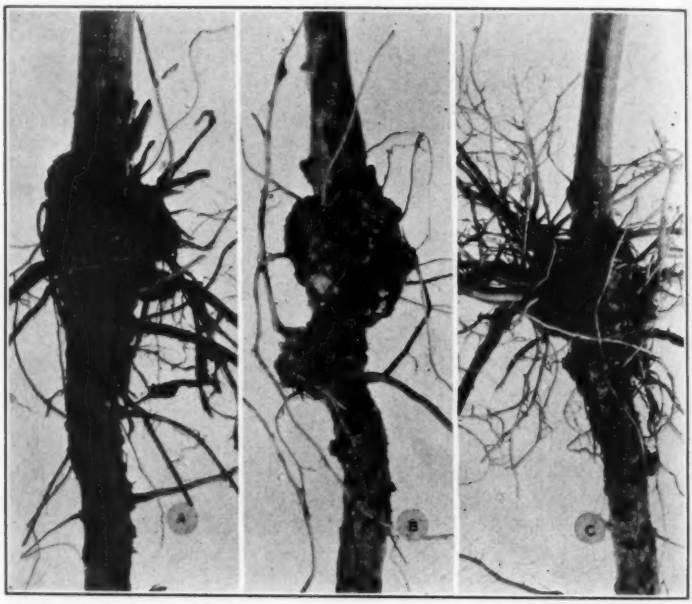

FIGURE 6.—A–C, Types of malformations on Winter Banana apple grafts resulting from inoculations with the apple organism. Natural size

In controlling malformations on the graft union, Hedgcock emphasized the importance of making well-fitted grafts. In 1921 the writer initiated experiments for the purpose of testing the effect on malformations of the use of well-matched and poorly matched grafts. In a general way it may be stated that the use of well-matched grafts lessened to some extent the number of malformations. Quite frequently, however, the well-matched grafts did not yield a larger percentage of clean trees than the poorly matched ones. Riker and Muncie (*14*) and other workers in this field have emphasized the importance of well-matched grafts as a means of controlling malformations. The writer, therefore, believing that perhaps there had not been sufficient contrast between well-matched and poorly fitted grafts in his previous experiments, repeated this experiment, making certain that the well-matched grafts were as perfect as it was possible

for an expert grafter to make them, and having the poorly fitted grafts made with the lower end of the scion blunt in some cases and pointed and extending over the cut surface of the seedling in others. The results of this experiment are given in Table 7.

TABLE 7.—*Results from comparative plantings of untreated poorly matched and well-matched grafts of Duchess apple*

[100 of each kind of graft]

Description of graft	Resulting trees			
	Total	Number smooth	Number galled	Per cent galled
Poorly matched:				
Lower end of scion blunt	67	1	66	98.5
Lower end of scion pointed	78	12	66	84.6
Well matched	71	8	63	88.7

It will be seen that an exceedingly large number of malformations, practically all on the graft union, resulted on all these grafts; and since 88.7 per cent appeared on the perfectly matched grafts, it is evident that the reliance on well-matched grafts alone as a control of malformations can be overemphasized.

DISCUSSION

As previously stated, the term "woolly knot" has been quite generally recognized as referring to a type of malformation characterized by a swelling more or less covered with fibrous or fleshy roots. Whether one designates this type of malformation as crown gall or as a form of hairy root is of small moment, as the terms have generally been used interchangeably. To prevent any possible confusion, however, the writer points to Figures 1 and 6 as illustrations of what he considers typical woolly-knot malformations. Malformations typical of what the writer terms "woolly knot" are found in illustrations designated as wound overgrowths by Riker and Keitt (*13, pl. 40, C*) and by Muncie (*7, pl. 2, B*). There is a tendency, perhaps unfortunate, to designate malformations exhibited on seedling roots as hairy root, while the same type of malformation on grafted trees would be generally termed "woolly knot." Whether this type of malformation is found on a 1-year-old seedling or on a 1-year-old grafted tree should not affect its status. Smith et al. (*17, pl. 18, fig. 1*) and Hedgcock (*2, pl. 6, fig. 5*) have also illustrated what the writer considers typical woolly knots. The malformations obtained by Smith et al. were secured by artificial inoculation of apple seedlings with what they termed the apple organism and were designated by them as apple hairy root. This type of malformation apparently is identical with those illustrated in Figure 1 of the present paper which resulted from inoculations with the apple organism.

The type of gall that Riker and Keitt (*13*) considered true bacterial gall is shown in their Plate 38, A–C. Muncie (*7, pl. 2, A*) also illustrated what he considered as typical galls, from which he isolated *Bacterium tumefaciens*. A very similar type of gall is shown

in Figure 2 of the present paper. This gall resulted from an inoculation on apple roots with the peach organism of Smith, isolated from a peach gall. It is presumably the only type of malformation on grafted apple trees that Riker and Keitt (*13*) and Muncie (*7*) recognize as being pathogenic in nature, and it is caused by the strain that is apparently identical with the peach and daisy strains of Smith et al. (*17*). Undoubtedly other strains may cause similar types of galls. It is seen that the dissimilarity between the rather smooth type of gall caused by the peach strain or similar strains and the woolly-knot type resulting from infections with what the writer terms the apple strain is quite marked. The distinction

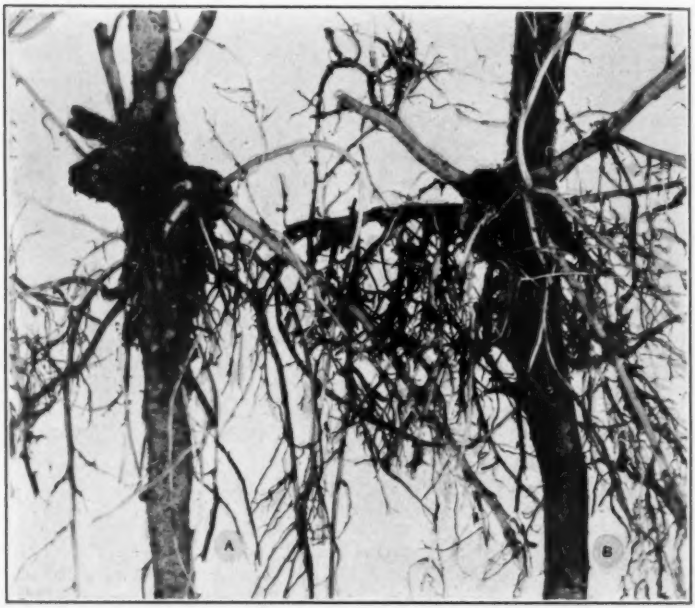

FIGURE 7.—A and B, Typical malformations on the scion wood of Yellow Transparent apple grafts resulting from inoculations with the apple organism. The roots from inoculations made on July 19, 1928, were more fibrous than those from inoculations made on August 20, 1928. (Compare with fig. 8, A–C.) Natural size

between these two types of galls is, of course, necessary for a proper comparison and interpretation of the results of these experiments, especially in so far as they are related to the investigations of others.

The experiments detailed in Table 1 were performed in a manner somewhat similar to one described by Muncie (*7*) except that Muncie used apple seedlings grown from seed planted the current year in soil that had been steamed, whereas the writer used 1-year-old transplanted seedlings in unsteamed soil. Muncie made inoculations with what was possibly the peach strain or at least a strain which produces similar results, whereas the writer used both apple and peach strains. Inoculations with the peach strain resulted in

9.15 and 29 per cent infection in Muncie's and in these experiments, respectively. However, in the writer's experiments the type of gall obtained was identical with the type that always resulted from inoculations with the apple organism. These galls were decidedly not of the type caused by the peach strain (fig. 2), and moreover when cultured they yielded, as was expected from their appearance, the apple organism. Muncie's experiments indicated that inocu-

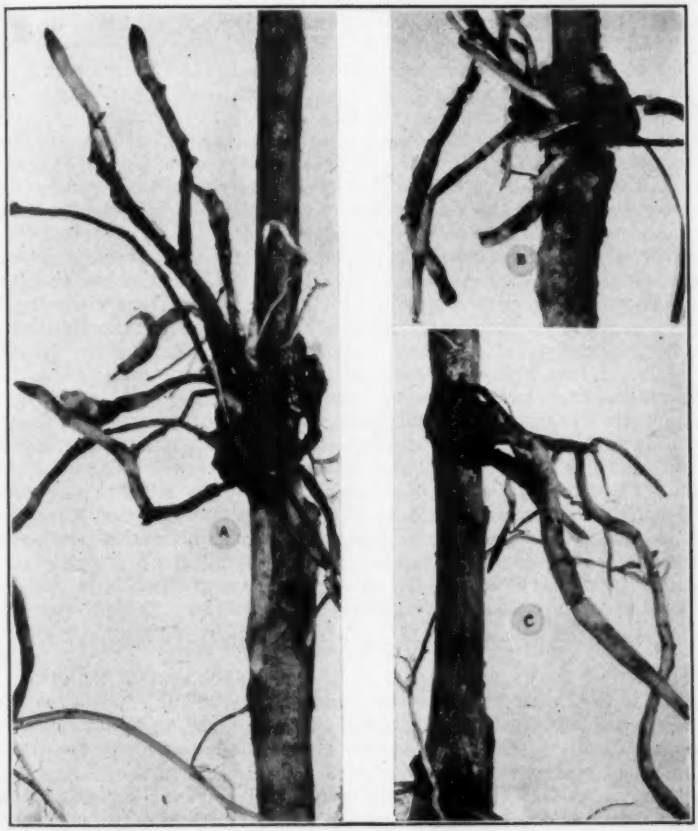

FIGURE 8.—A–C, Fleshy roots from malformations secured as a result of inoculations made on August 20, 1928, on scion wood of Yellow Transparent apple grafts. (Compare with fig. 7, A and B.) Natural size

lations with the peach strain resulted in the "woolly-knot form of hairy root." No report of isolations is given, and it is perhaps reasonable to assume that the woolly knots obtained in Muncie's experiments would have yielded in culture the apple organism and not the original organism. On the other hand, it might be assumed that the peach organism was actually present in the galls obtained by the writer and the culturing technic failed to reveal it. This

latter assumption loses force, however, when it is recalled that the galls were typical of those invariably obtained with the apple organism. Still, the fact that the controls in each of these experiments were practically free of infection must be considered. To regard the natural infections on the seedlings inoculated with the peach organism in the writer's experiments as fortuitous might be permissible; to apply the same conception to Muncie's experiments, in steamed soil, is permissible only with qualifications. In view of the results of the subsequent experiments reported here, wherein inoculations on apple grafts with the peach organism practically failed of infection, whereas those with the apple strain resulted in high percentages of infection, it is considered that, regardless of any interpretation of this particular experiment, the results and interpretations of the other experiments are not materially influenced by this one. This experiment is reported not as furnishing evidence but rather as illustrating how diametrically opposed conclusions can be drawn from the similar results of similar experiments.

Riker et al. (*11*) in a recent reference to Muncie (*7*), stated that he considered that the "woolly-knot form is a manifestation of crown-gall infection." It seems desirable to direct attention to the fact that Muncie's experiments from which these conclusions were drawn were on apple seedlings and not on apple grafts and that galls on apple grafts, typical of woolly knots, were illustrated by Muncie (*7*, *pl. 2*, *B*) as being the type of malformations "from which *Ps. tumefaciens* was not recovered" and hence considered by him nonpathogenic overgrowths, and finally, that it is quite possible, as has been stated, that the malformations which Muncie secured by means of inoculations with the peach strain actually resulted from natural infections of the apple organism and not from the original inoculum. The conclusion drawn by Muncie that "the woolly-knot form is a manifestation of crown-gall infection" when it occurs on apple seedlings but that "the abundant development of fibrous roots from an overgrowth at the union of piece-root grafted trees is not a reliable index of crown-gall infection" may not be tenable.

CAUSE OF THE WOOLLY-KNOT TYPE OF MALFORMATION

The writer (*16*) has reported on the rather consistent isolation of the apple organism from the woolly-knot type of malformations and on the pathogenicity of this strain of the crown-gall organism on apple shoots as well as on other hosts. The fact that so many isolations were made and reported in previous experiments is the reason why only comparatively few isolations were made in these investigations.

Evidence shown in Table 5, supplemented by that in the other tables, consistently shows that the apple organism is pathogenic on apple roots at the graft union and causes the type of malformation most commonly encountered in the root-grafted apple nursery. It is considered that the experiments have contained sufficiently large numbers, have been repeated often enough, and have yielded results so consistent as to afford conclusive interpretations of the results.

In the recent report of Riker et al. (*11*) concerning their inoculations on underground parts of apple shoots with strains of bacteria considered to "closely resemble" *Bacterium tumefaciens*, it is noted that

these strains, which previously had failed to cause infection when inoculated into tomato stems, did result in infections, characterized as "enlargements which occurred at the bases of these excessive root developments * * *," when inoculated on the underground shoots of apple. It is quite possible that these so-called root-stimulating organisms of Riker et al. (*11*) may play a leading rôle in connection with the woolly-knot malformations on apple grafts or seedlings. Regardless of the question of the specific identity of the organisms used by these investigators, the writer considers that their recent investigations furnish strong evidence supporting the pathogenic conception of woolly-knot malformations.

STRAINS OF BACTERIUM TUMEFACIENS

Since a prolonged discussion of the strains of *Bacterium tumefaciens* is not within the scope of this paper, reference is made to Smith et al. (*17*). Patel (*9*) noted the occurrence of what he termed nonpathogenic strains of *Bacterium tumefaciens*. Riker et al. (*11*) reported the isolation of "* * * organisms which were similar to *Bact. tumefaciens* in certain routine diagnostic characters * * *" from "a large majority of the naturally occurring malformations" and noted that this organism is capable of causing a reaction on the underground shoots of apples. It is quite probable that this organism is closely similar to, if not identical with, the one referred to by the writer (*16*) as the apple strain. The fact that Patel (*10*), however, was unable to secure infection on the apple with what he termed some "nonpathogenic" strains of *Bact. tumefaciens* does not support this impression. It is further noted that Riker et al. (*11*) reported that in their previous experiments these organisms "failed to induce positive evidences of infection when inoculated through punctures into stems of * * * apple." The interpretation to be placed on the word "positive" (evidence) is mootable. However, the condition of the host, as well as the technic used, the virulence of the culture, and other factors may account for some discrepancies. Stapp (*19*) and other European workers also recognized widely divergent strains of the crown-gall organism. As stated previously, the writer (*16*) considers that there are just as wide divergences in other species of bacteria.

SUSCEPTIBILITY OF NEWLY MADE GRAFTS TO INFECTION

As stated, the condition of the hosts, the technic, the age and virulence of the inoculum, the temperature and moisture relations, and numerous other factors are of especial importance in experiments with the crown-gall organisms.

Hedgcock (*2*) expressed the opinion that most apple-root grafts become diseased with crown gall apparently at the time of the formation of the callus. Riker and Keitt (*13*) concluded, as a result of their experiments in which the callused grafts were immersed in the inoculum, that the freshly produced callus is not an open infection court for the crown-gall organism. In view of the fact that the writer had secured a large measure of control of the woolly-knot malformation by disinfecting the scions and stock before grafting, an experiment was planned, as stated previously, to determine at just what time subsequent to grafting the grafts were most subject to infection. Inoculations on grafts were made with the peach and

the apple strains, not only subsequently but also previously to callus formation. This experiment (Table 5) gave in part the same results as the one performed by Riker and Keitt but admitted of a different interpretation. Here the peach strain failed to cause infection, just as did the strain that Riker and Keitt used. The freshly formed callus is not an "infection court" for the peach or similar organisms, as Riker and Keitt (*13*) rightly concluded, but it is extremely susceptible tissue for the apple organism. The evidence furnished by this experiment indicated that most infections occur during the precallusing or early-callusing period, rather than after the callus has formed. Experiments similarly conducted in the season of 1928 completely confirmed this conclusion and again demonstrated that the apple strain was the cause of the resulting malformations. The illustrations in Figure 6 represent malformations typical of those secured by inoculation with the apple organism at the time of grafting. This type of malformation previously yielded the apple strain in culture quite consistently. The controls were practically clean. It is believed that this evidence, offered as proof that most infections occur previous to callus formation, explains why surface disinfection of the seedling stocks and scions affords some measure of control.

It should be noted, however, that Riker and Keitt (*13, p. 772*) reported negative results in controlling malformations by means of surface disinfection of stocks and scions. The writer's results are not in conformity with those reported by these investigators, who also dipped the freshly made grafts in antiseptics. Perhaps this discrepancy may be explained in part by reference to the writer's (*16*) previous statement that he did not consider the malformations illustrated by Riker and Keitt (*13, pl. 45*) or by Muncie (*7, pl. 3, b*) as being typical of galls most commonly encountered in the root-graft apple nursery.

WELL-MATCHED GRAFTS AS A MEANS OF CONTROL

The writer's experiments have in the main indicated that careful attention to making well-matched grafts may result in a decrease in the number of malformations, but the data given in Table 7 indicate that, under conditions favorable for natural infection, the importance of well-matched grafts as a means of control can be overemphasized.

GROWTH AND NUTRITIONAL CONDITIONS

In the literature relating to crown-gall inoculations there has been a tendency to give considerable attention to the so-called vigor of the host.

In seeking an explanation as to why woolly-knot malformations were not readily found until the fall of the year on grafts planted the current year, and in view of the fact that large galls are found on very small as well as on large yearling trees the thought occurred that perhaps the question of nutrition at the point of infection might be more important than that of vigor or rapidity of growth. The experiments detailed in Table 6 were performed in part to test this hypothesis. Since the malformations on the grafts inoculated August 20 were equally as large as those obtained on the grafts inoculated on July 19, one month earlier, there is an indication that the increased supply of elaborated food materials at the point of inoculation in

late August may be a very important factor in securing infection and in influencing the size of the gall. Although numerous other factors necessarily should be considered in this connection, additional experiments which are so preliminary in nature that it is not deemed advisable to report them here in detail afford evidence to support this hypothesis. In brief, seedling apple trees of the current season's growth have been inoculated with the apple strain of the crown-gall organism by means of needle punctures at a point immediately above the axillary bud. The area immediately below the leaf petiole has been girdled by means of a sharp scalpel in some cases; in other cases the analogous areas have not been girdled. From the results of one season's experiments only, there is evidence that infections have been secured more readily in those areas that were girdled below the leaf petiole.

CONCLUSIONS

Because of the nature of the subject matter in the "Discussion," a recapitulation of the salient points produced by these experiments may be helpful, especially in correlating the interpretations of the results of these experiments with those obtained by other investigators.

The primary purpose of the studies reported in this paper has been to discover the cause of malformations known as woolly knots prevalent on root-grafted apple trees. The question raised as to the cause of this type of malformation by Riker and Keitt (*12*, *13*), by Melhus (*4*), and by Muncie (*7*), and the conclusion of these writers that the problem of malformations on apple grafts was one dealing primarily with nonbacterial wound overgrowths, made it advisable not only to perform experiments on the aboveground and underground tissues of the apple but to give special attention to the graft union itself. At this point of contact between the seedling root and scion are found the majority of the malformations. The bacterial organism, previously reported by Siegler (*16*) as probably being identical with the apple strain of Smith and others (*17*), was isolated rather consistently from the malformations in question and was found to exhibit a degree of parasitism on apple shoots and other hosts. A limited number of inoculations with another organism, apparently the peach strain of Smith et al., were also made. This organism causes a comparatively smooth, rounded type of malformation on apple roots and apparently is the only type of gall on root-grafted apple trees considered by Riker and Keitt (*13*) and Muncie (*7*) as pathogenic. Although Muncie (*7*) credited a certain amount of woolly-knot hairy root with being infectious on apple seedlings, this conclusion is subject to several interpretations which have been discussed. Furthermore, Riker and Keitt (*13*) reported: "In cases in which roots have been observed in association with a crown gall they have been found to occur in connection with wound overgrowth that was adjacent to the gall." Thus they concluded that the hairy roots were in fact not a manifestation of infection, and at that time interpreted Muncie's results to be in conformity with their conception that the hairy roots arise from wound overgrowths. Recently, however, Riker et al. (*11*) have given a different interpretation of Muncie's results and now apparently accept his conclusion, referring to apple seedlings, that the "woolly-knot form is a manifestation of

crown-gall infection." Regardless of the above interpretations and those discussed previously, the cause of the woolly-knot malformations on the graft union has been the chief concern of these experiments.

In order to avoid confusion of issues, it is believed that it is essential to recognize the practical synonymy of the terms "crown gall" and "hairy root" and the identity of the distinct types of galls produced by the peach or closely related strains of the crown-gall organism and by the so-called apple strain. Despite differences between these so-called strains, it is a matter of judgment whether these strains should be considered as worthy of specific rank.

As Riker and Keitt (*13*) noted, Smith et al. (*17*) performed only a limited number of experiments on the apple and did not produce evidence sufficient to reach a conclusion concerning the identity of the woolly-knot malformations. In the inoculation experiments of Smith et al. (*17*) with the apple organism the checks, when used, became infected all too frequently, and no inoculations were made on the graft union; with the peach organism, inoculations on the roots were practically failures. On the other hand, while Smith et al.(*17*) did not offer conclusive proof in this case, their experiments furnished certain leads which should not be disregarded.

The assumption that the organism referred to in these experiments as the apple strain is identical with the apple strain of *Bacterium tumefaciens* of Smith et al. (*17*) appears warranted. It is probable that other workers have also isolated this organism but have regarded it as a nonpathogenic strain of *Bact. tumefaciens*. Recently Riker et al. (*11*) reported the results of inoculations on underground stems of apple with what was probably an organism previously regarded as a nonpathogenic strain of *Bact. tumefaciens*. A root-stimulating effect was noted. These latest experiments of Riker et al. (*11*) are in agreement with some of the experiments reported here and are thought to confirm the evidence that woolly-knot malformations are caused by bacteria, regardless of the specific identity of the organism.

The fact that the freshly made graft is susceptible at the union to infection with the apple organism is apparently proved by the evidence furnished by these experiments. That the graft union after callus has formed is an infection court to a limited extent for the apple organism is also indicated by the evidence herein shown. The conclusion of Riker and Keitt (*13*) that the graft union is not an open court for infection for the crown-gall organism holds true for certain strains only but not for the apple strain, which was not reported as being used by these investigators.

The evidence that most of the infections occur during the time the grafts are callusing supports the opinion expressed by Hedgcock (*2*). It also offers a reasonable explanation for the control of malformations when the scions and stock are disinfected previous to grafting.

There is some evidence suggesting that the amount of nutrition at the point of inoculation is more important than a condition of rapidly growing tissue, in the amount and kind of infection secured. If this is correct, an explanation as to why the freshly made graft is especially susceptible to infection with the apple organism is offered. The seedling stock is, of course, comparatively rich in food reserves at the time of grafting.

There was a marked difference in the character of the roots springing from malformations secured as a result of inoculations made in

July and August, respectively. The excessive fleshiness of the roots resulting from inoculations in late summer may be due, among other causes, to a certain degree of immaturity, to a mechanical action, or even to a physiological action whereby only the downward passage of food material is permitted or is active. These are questions not especially germane to the end results of these experiments.

The facts that woolly-knot malformations were produced on graft unions by inoculations with the apple strain of *Bacterium tumefaciens* in a large series of experiments and with the use of a number of varieties, that these woolly knots consistently yield this organism in cultures the validity of which have been proved by reinoculations, and that the control grafts in each of these series have remained practically free of malformations, are considered proof sufficient to warrant the conclusion that this type of malformation is pathogenic and is caused by a bacterial organism which can be tentatively referred to as the apple strain of the crown-gall organism. These conclusions are supported by results obtained on apple seedlings.

SUMMARY

In this paper are reported experiments in which apple grafts and seedlings have been inoculated with an organism tentatively referred to as the apple strain of the crown-gall organism (*Bacterium tumefaciens*). This is probably the same organism that Smith et al. isolated from malformations on apple roots, and because of the wide divergence in strains of the crown-gall organism it is deemed advisable to continue the designation of this organism as the apple strain. This so-called apple strain causes the woolly-knot type of crown gall or hairy root prevalent on root-grafted apple trees.

It is probable that this apple strain is identical or at least closely related to the so-called nonpathogenic strains of *Bacterium tumefaciens* of other workers, since those strains have produced similar results when inoculated on the below-ground parts of apple shoots.

When apple grafts were immersed in an inoculum of the apple organism previous to callus formation, abundant infection occurred; when immersed in the inoculum after callus had formed, infection was not so abundant. One other strain of the crown-gall organism failed to cause infection when inoculated at these periods.

The reason why surface disinfection of scions and stock previous to grafting affords some measure of control may be due to the aseptic condition of the grafts at a time when they are susceptible to infection.

Limited experiments indicate that the nutritional condition at the point of inoculation may be a factor of greater importance than the condition of rapid growth, in the amount and kind of infection secured.

The woolly-knot type of crown gall on apple grafts caused by the apple organism is quite distinct from the comparatively smooth type of gall with slight or no root developments caused by certain other strains of the crown-gall organisms.

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THE MEAN AND VARIABILTY AS AFFECTED BY CONTINUOUS SELECTION FOR COMPOSITION IN CORN[1]

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INTRODUCTION

The effectiveness of selection in cross-fertilized crops such as maize may be explained upon the following principles: (1) There are heritable variations existing among the different individuals of the population at the beginning; (2) there is a gradual elimination of the undesirable type because the selected types consist of more desirable and fewer undesirable individuals in each generation; (3) desirable mutations which may occur are retained and caused to combine with the desirable factors present; and (4) recombination of the desirable factors produces more desirable types.

Selection for a given type not only tends to bring the population to that type but is expected to decrease the variability. This decrease in variability of the population is brought about by a reduction in the percentage of heterozygous individuals. After the population becomes homozygous for the selected character, no further reduction in variability through selection can be expected. The variability that still remains is attributed to environment, upon which selection has no influence.

It is the purpose of this paper to present the effect of 28 years of continuous selection for composition in maize upon the mean and the variability of the selected character.

MATERIAL

In 1896 a series of breeding experiments was begun at the Illinois Agricultural Experiment Station to determine whether the chemical composition of corn could be influenced by selection (*8*).[3] One hundred and sixty-three ears of a variety known as Burr's White were used as the foundation stock, from which selections were made in four different directions, namely, for high oil, low oil, high protein, and low protein.

These four strains were carried on in the same way. In the high protein, for example, the 24 ears highest in protein were selected for seed and planted in an isolated plot, each ear in a separate row. These rows were harvested separately and the seed for the next crop selected from the ears which were found to be highest in protein. Nine years later the system was modified somewhat in an attempt to prevent loss of vigor by inbreeding. Alternate rows were detas-

[1] Received for publication Mar. 9, 1929; issued September, 1929.

[2] The writer wishes to thank Dr. L. H. Smith for suggesting the problem. To Dr. C. M. Woodworth, under whose direction these studies were made, the writer expresses sincere thanks for suggestions in analyzing the data and for valuable assistance in the preparation of this paper.

[3] Reference is made by number (italic) to "Literature cited," p. 475.

seled and seed was selected only from the highest yielding detasseled rows. In 1921 this system was again modified to reduce the amount of inbreeding. Two seed ears were taken from each of the detasseled rows regardless of yield. The high-oil, low-oil, and low-protein tests were similarly conducted, selection being made each year of ears highest in oil, lowest in oil, and lowest in protein, respectively.

The analytical methods employed have been described in detail in Illinois Agricultural Experiment Station Bulletins 43 and 53 (*6*, *7*).

EFFECT OF SELECTION FOR PROTEIN CONTENT

The effect of selection for protein content in the corn grain is shown in Figure 1. The average protein content which was 10.92 in

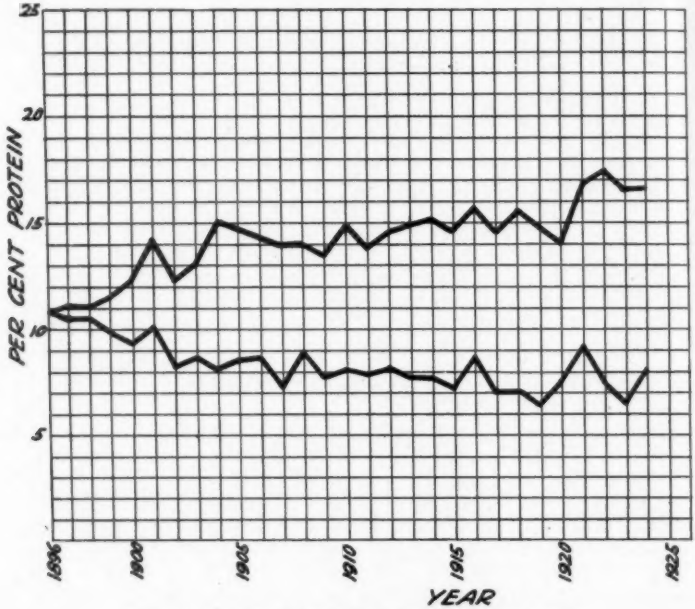

FIGURE 1.—Progress of high-protein and low-protein corn breeding

1896 had in 1924 been increased to 16.60 per cent in the high-protein strain and had been decreased to 8.38 per cent in low-protein strain. This is a difference of 8.22 per cent. When measured by the best fitting straight lines, the difference is 9.49 per cent. When compared with the original variety from which the strains were selected, the proportional increase is 50.01 per cent and the proportional decrease is 23.26 per cent.

EFFECT OF SELECTION FOR OIL CONTENT

From an average of 4.70 per cent in 1896, the oil content had been increased to 9.86 in the high-oil strain and decreased to 1.51 per cent

in the low-oil strain in 1924. This is a difference of 8.35 per cent. When measured by the best fitting straight lines, the difference is 8.85 per cent. Relatively, selection has been much more effective in bringing about a change in oil content than in protein content. When compared with the original variety, the proportional increase in oil is 109.79 per cent and the proportional decrease is 67.87 per cent. (Fig. 2.)

It is very probable that a more rapid shift in type with respect to both the oil and protein would have been obtained if only the ears

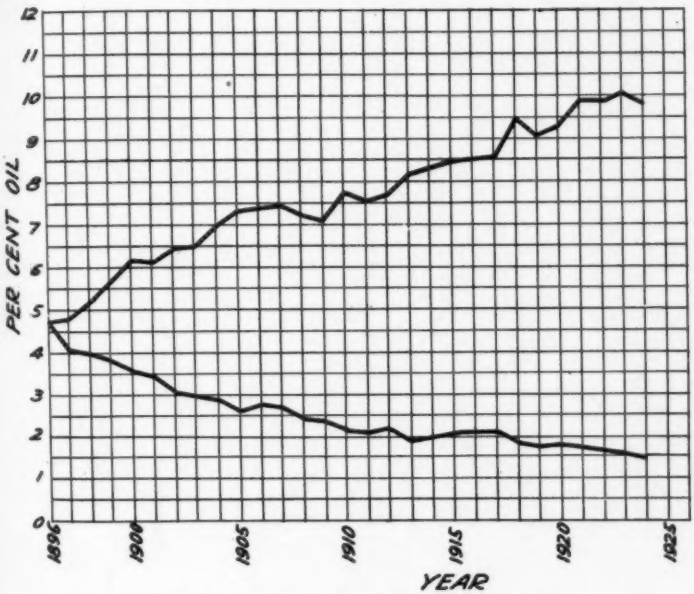

FIGURE 2.—Progress of high-oil and low-oil corn breeding

that were highest and lowest in protein or in oil had been selected for seed, regardless of the yielding ability of the detasseled rows. On the other hand, if selection had been carried on without taking yield into account, inbreeding might have resulted in such a loss of vigor that it would have been impossible to continue the strains. When it is remembered that the original purpose of the experiment was to produce good-yielding strains having the desired composition, any great reduction in yield would have defeated the purpose.

PEDIGREES

Graphic presentations of the pedigrees of the strains of corn used in this work follow.

PEDIGREE OF ILLINOIS LOW-PROTEIN CORN

GENERATION NUMBER																											
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
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[1] 06	10	14	03	06	02	15	09	20	10	04	08	10										10	12	08	10	10	10
													18	10	18	02	08	08	12	08	18						11
												22										14	14	12	12	12	12
													24	14	22	14	22	12	14	24	20						13
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																											21
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																											23
																									24	24	24

[1] These numbers, preceded by the generation numbers, denote the seed ear numbers.

PEDIGREE OF ILLINOIS HIGH-PROTEIN CORN

GENERATION NUMBER

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
																											01
																									02	02	02
																											03
																									04	04	04
																											05
																									06	06	06
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																	08	16	02	12	04	08					11
												06	10	10	14	06							06	10	12	12	12
[1] 21	07	23	13	07	07	17	18	22	22	12	24						20	20	06	18	06	14					13
												18	14	14	24	12							10	12	14	14	14
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[1] These numbers, preceded by the generation numbers, denote the seed ear numbers.

PEDIGREE OF ILLINOIS HIGH-OIL CORN

GENERATION NUMBER

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
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[1] These numbers, preceded by the generation numbers, denote the seed ear numbers.

PEDIGREE OF ILLINOIS LOW-OIL CORN

PEDIGREE OF ILLINOIS LOW-OIL CORN

GENERATION NUMBER

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
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[1] 10	06	10	07	04	05	13	07	10								14	12	10	08	06	04	08					12
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[1] These numbers, preceded by the generation numbers, denote the seed ear numbers.

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A study of the pedigrees of the four strains shows that there has been a rapid elimination of lines until the four strains are now represented by only a single ear each of the original seed stock. The high-protein strain traces back to ear 121 which on analysis showed 12.28 per cent protein and 3.99 per cent oil. This is slightly below the average composition of 12.54 per cent for the stock seed ears but considerably above the average of the original 163 ears, 10.92 per cent. The low-protein strain goes back to ear 106, which on analysis yielded 8.25 per cent protein and 4.81 per cent oil. The average for the stock seed ears of the low-protein strain was 8.96 per cent. The high-oil strain goes back to ear 111, which on analysis showed 5.65 per cent oil and 10.82 per cent protein. The average for the stock seed ears for the high oil was 5.33 per cent as compared to 4.70 per cent, the average for the original 163 ears. The low-oil strain traces back to ear 110, which when analyzed was found to contain 4.10 per cent oil and 11.13 per cent protein. The average percentage of oil for the stock seed ears was 4.04. Thus, in the twenty-eighth year of selection all of the 96 ears of the four strains trace back to 4 ears of the original Burr's White.

Even though the ear has been used as the unit of selection and the history of the strains traced through the female side only, the reduction of the ancestry to a single ear for each strain indicates that the strains at the present date are likely to be more nearly homozygous than was the original material from which they came. Further evidence that the strains are more homozygous than open-pollinated varieties is furnished by selfing the strains. It has been shown at this station that upon selfing, a condition of uniformity is reached more quickly than with open-pollinated varieties. Although the individual inbred lines coming from any one of the strains differ among themselves in composition, they are always significantly different from any of the inbreds coming from the other strains.

That there is still some heterozygosity left in the individual within the strains is indicated by the fact that there is a reduction in vigor upon selfing.

East (*2*), in fitting curves to the data for the first 10 generations of selection, found that at first the curve was concave, showing great progress, later convex, showing that progress became slower, and at last horizontal, indicating that no more progress would result from selection. An inspection of Figures 1 and 2 presented here will show that, contrary to East's results, there has been considerable progress since the tenth generation for all of the strains with the possible exception of the Illinois low protein. The Illinois high-oil strain has shown greater progress since the tenth generation than it did before.

It does not seem possible to predict a limit to the progress that selection will make in the Illinois high-oil and the Illinois high-protein strains by the application of curves to the data at hand. Apparently the low-protein strain has made but little change in the last 20 years. If the average protein content for the years beginning with 1902 be compared with the protein content of ear 106, to which the strain now traces, no significant difference appears.

The low-oil strain is approaching a physiological limit. The greater percentage of the oil in a grain of corn is contained in the germ (*9*). Hence, in selecting for low-oil content the size of the germ has been decreased both absolutely and relatively in comparison with the size

of the endosperm. In the ear containing the lowest percentage of oil on record, namely, 0.69 per cent, 80 per cent of the grains were germless. The necessity of using ears having grains that will germinate naturally tends, therefore, to check the progress of selection, and eventually may stop it altogether.

EFFECT OF SELECTION UPON VARIABILITY

It seems to be accepted by most biologists that selection for a given character in a cross-fertilized crop like corn leads to a lower variability. That this reduction in variability may have its limits is brought out by Davenport (*1*) when he states that selection in a cross-fertilized crop such as corn simply shifts the type but does not appreciably change the variability unless a physiological limit is reached. As evidence for this belief, he cites Karl Pearson as stating that 10 to 13 per cent reduction in variability obtained by the selection of two parents is almost the limit that can be reached, even if the complete ancestry

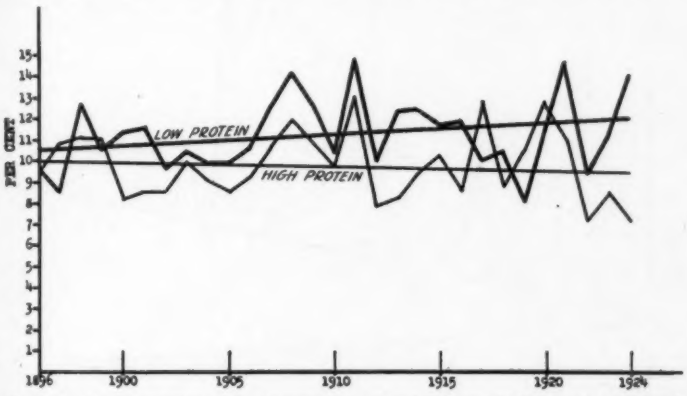

FIGURE 3.—Yearly variations in protein content of corn and their straight-line trends as measured by the coefficient of variation

had been selected. Davenport also uses the first eight years' data on the Illinois "chemical" strains of corn, employing the coefficient of variation as a measure of variability, and states that there is no significant change in variability. It is of interest to see how the matter stands after the results of 20 more years of continued selection have been secured.

VARIABILITY MEASURED BY THE COEFFICIENT OF VARIATION

PROTEIN

The effect of selection upon the variability in protein content as measured by the coefficient of variation from the beginning of the experiment through the 28 years of selection can be seen in Figure 3. Although the yearly variations as measured by this coefficient are rather large, there are decided trends that run in directions opposite to the line of selection. As selection leads to a low mean, the variability increases, and vice versa. As measured by the best fitting lines, the variability has increased 14.05 per cent in the low-protein strain over that at the beginning and decreased 4.83 per cent in the high-protein.

Oil

Similar results have been obtained for the oil content; only here the divergence is more marked. The variability, according to the coefficient of variability, has increased 69.84 per cent for the low-oil strain and decreased 23.21 per cent for the high-oil strain. (Fig. 4.)

It appears from these results that selection can change variability, as measured by the coefficient of variation, more than 13 per cent and that the variability may either increase or decrease, depending upon the magnitude of the mean. Although the coefficient of variation has been the most commonly used means of comparing variability when different types of variation are involved in the comparison, it is

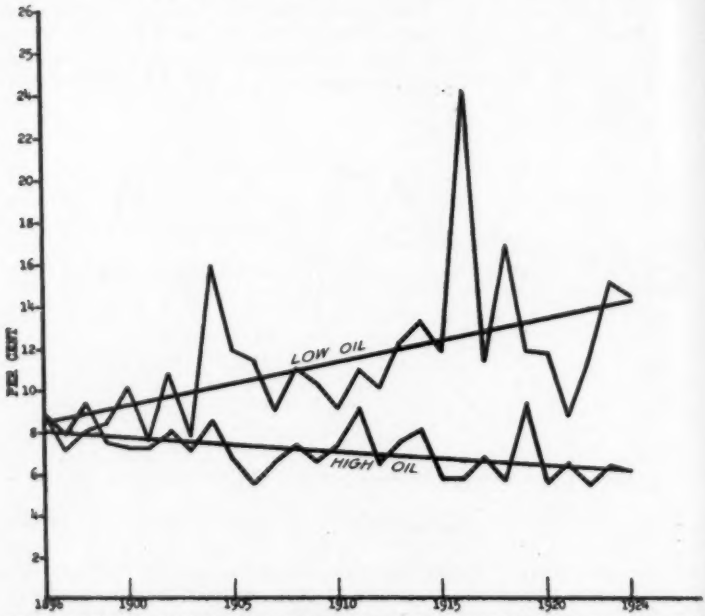

FIGURE 4.—Yearly variations in the oil content of corn and their straight-line trends as measured by the coefficient of variation

recognized to have decided limitations. Pearl (*12*) states that, " . . . the coefficient of variation has never been an entirely satisfactory constant to biologists, at least," and also that (*11, p. 275*) "one should always remember that this constant simply measures the degree of scatter of the distribution in relation to the mean value of the thing varying." Such a relation may have a real and significant meaning but sometimes it does not have, for reasons inherent in the nature of the facts themselves.

VARIABILITY MEASURED BY THE STANDARD DEVIATION

The standard deviation has usually been accepted as the standard method of measuring in absolute terms the degree of variability. It has the advantage that it is a constant of the mathematical formula for the curve of variation representing the distribution of a population.

Protein

The variability for protein content in the Illinois high-protein and Illinois low-protein strains, as measured by the standard deviation (fig. 5), shows trends opposite to those obtained when the coefficient of variation is used to measure the variation. Although showing considerable yearly fluctuation, the variability tends to move in the same direction as the mean; e. g., the high-protein strain is more variable than the low-protein strain for protein content. During the

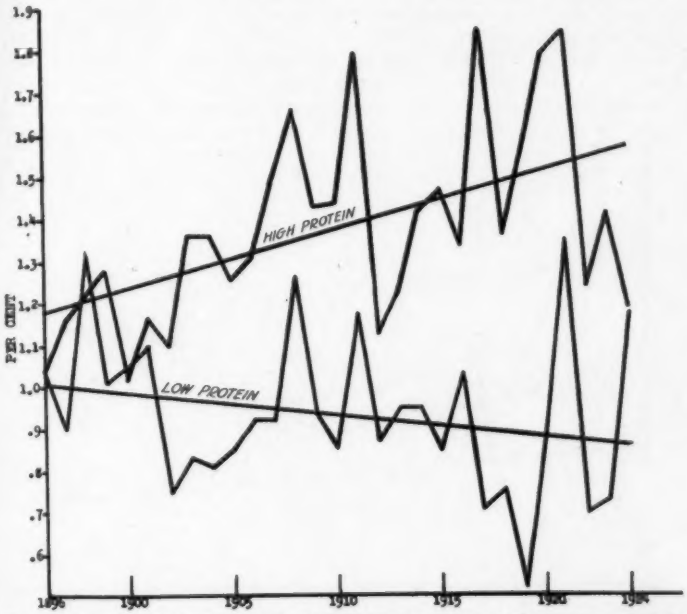

FIGURE 5.—Yearly variations in protein content of corn and their straight-line trends as measured by the standard deviation, 1896–1924

course of the experiments an increase in variability, according to this index, of 32.97 per cent for the high-protein strain and a decrease of 14.71 per cent for the low-protein strain have been obtained.

Oil

The variability measured by the standard deviation for the oil content shows similar results. (Fig. 6.) Here also the variability is greater in the strain having the higher content. An increase in variability of 50.95 per cent in the high-oil strain and a decrease of 36.44 per cent in the low-oil have been obtained. Again, the greater divergence in variability occurs between the oil strains.

When the standard deviation is used as a measure of variability, greater changes are exhibited than when the coefficient of variation is employed except for the low-oil strain. However, the standard

deviation has its limitations also. It can not be used to compare variability except where like things are measured in like units.

VARIABILITY MEASURED BY THE WEINBERG FORMULA

Weinberg (*14*) has recently proposed a method for measuring variation which is free from most of the limitations of the coefficient of variation and standard deviation. The coefficient $W =$

$$\frac{\sigma\sqrt{M_n - M_o}}{\sqrt{(M_a - M_o)(M_n - M_a)}}$$

when M_n is the highest value in the distribution, M_o is the lowest value in the distribution, M_a is the mean value of all variants. The denominator of the formula measures roughly the skewness of the variability curve, while the second term in the numerator measures

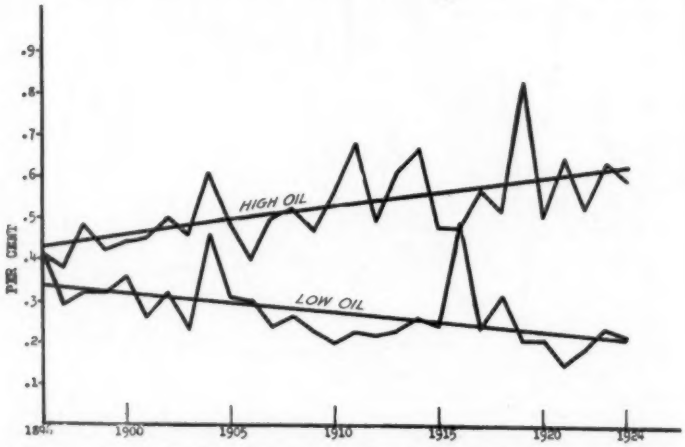

FIGURE 6.—Yearly variations in oil content of corn and their straight-line trends as measured by the standard deviation, 1896–1924

the range. The greater the skewness or the wider the range, or both, the greater the variability. The coefficient thus obtained is not affected by the magnitude of the mean as is the coefficient of variation, nor is it limited by unlike material or unlike units of measure as is the standard deviation. Like the coefficient of variation, however, it expresses variation as an abstract figure.

In Table 1 Weinberg's method is compared with the coefficient of variation for measuring variation by means of two examples. In example 1 the mean is 5, the standard deviation, 2.582, and the coefficient of variation is 51.64 per cent. By the Weinberg method the variation is 1.83. In example 2 each class is 10 units higher. The mean is 15, the standard deviation is the same as in example 1, but the coefficient of variation is 17.21 per cent. Variation, as expressed by Weinberg's formula, is the same as in example 1. It is believed that for comparative purposes the Weinberg formula gives a better conception of variability than does the coefficient of variation.

TABLE 1.—*Comparison of coefficient of variation with Weinberg's formula for measuring variation*

Example 1		Example 2	
Class	f	Class	f
1	1	11	1
2	1	12	1
3	1	13	1
4	1	14	1
5	1	15	1
6	1	16	1
7	1	17	1
8	1	18	1
9	1	19	1
Mean=5		Mean=15	
S. D. =2.582		S. D. =2.582	
C. V.=51.64%		C. V.=17.21%	
W.=1.83		W.=1.83	

PROTEIN

The effect of selection upon variability for protein content, as measured by the Weinberg formula (fig. 7), is similar to the results

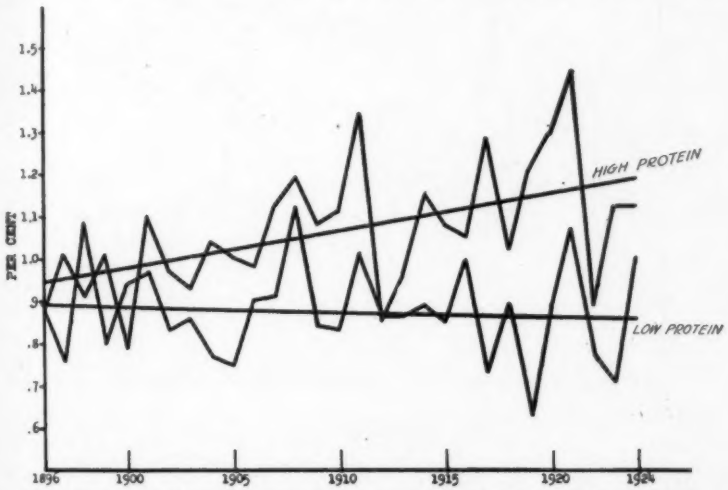

FIGURE 7.—Yearly variations in protein content of corn and their straight-line trends as measured by Weinberg's formula, 1896–1924

obtained when the standard deviation is used in that variability increases as selection leads to a high mean and decreases as selection leads to a low mean. The increase in variability for the high-protein strain was 25.49 per cent. However, the decrease in variability for the low-protein strain was only 3.94 per cent.

OIL

Similar results were obtained for variability in oil content of the oil strains. (Fig. 8.) The increase in the high-oil strain was 23.97 per cent and the decrease in the low-oil strain was 30.18 per cent. Again, the divergence in variability is greater between the oil strains than between the protein strains.

VARIABILITY MEASURED BY THE MODAL CLASS

Since the type of the population is represented by the modal class, it is of interest to know what proportion of the population resides within the modal class as selection continues.

A graphical representation of the modal classes in respect to composition for the four different strains taken at periodic intervals is seen in Figure 9. The blocks represent the relative proportion of the population residing in the modal class for the respective years. Although there is considerable fluctuation, it can be seen that the trend is downward for the high-oil and the high-protein strains, i. e., as selection leads toward a higher mean fewer and fewer individuals of the population reside within the modal class. In the low-oil strain there is a decided trend upward. The trend in the

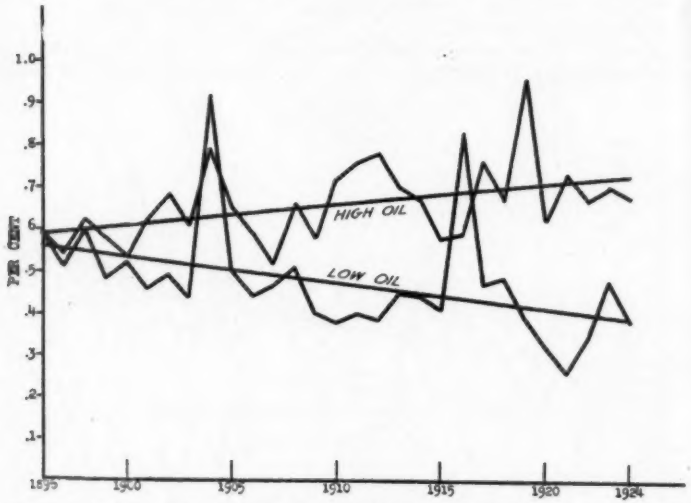

FIGURE 8.—Yearly variations in oil content of corn and their straight-line trends as measured by Weinberg's formula, 1896–1924

low-protein strain is slightly upward. Hence, selection for a low mean increases the percentage of individuals lying in the modal class.

The percentage of the population in the modal class may be taken as a rough measure of the uniformity, and, correspondingly, the percentage outside of the modal class as an expression of variability. In general, the greater the percentage of the population lying in the modal class the less the variability, and vice versa. This variability may be expressed in the form of a ratio $\frac{100-Y}{Y}$, where Y is the percentage of the population lying in the modal class. The smaller Y is the greater the ratio; hence, the greater the variability.

Variability may also be expressed by the inverted straight-line trend for the modal classes. For the purposes of this work such trends were determined by the use of the formulas $\frac{100-Y_o}{Y_o}=X_o$

and $\frac{100-Y_d}{Y_d}=X_d$ when Y_o is the origin and Y_d the destination of the best fitting straight line for the modal classes, and X_o and X_d are the origin and destination, respectively, for the lines to be determined. The quotient thus obtained may be called the extramodal coefficient because it takes into account the population outside of the modal class. The trends thus obtained (figs. 10, 11, 12, 13) indicate that variability becomes less as a low mean is approached and greater as a high mean is approached. The method is empirical and therefore has the limitations that most empirical formulas have.

COMPARISON OF THE DIFFERENT MEAURES OF VARIABILITY

A comparison of the four different measures of variability discussed above for each of the four strains is shown in Figures 10, 11, 12, and 13. The standard deviation and the coefficient obtained by the Weinberg method are multiplied by 10 so that they may be plotted on a common scale with the other measures. It is to be noted that

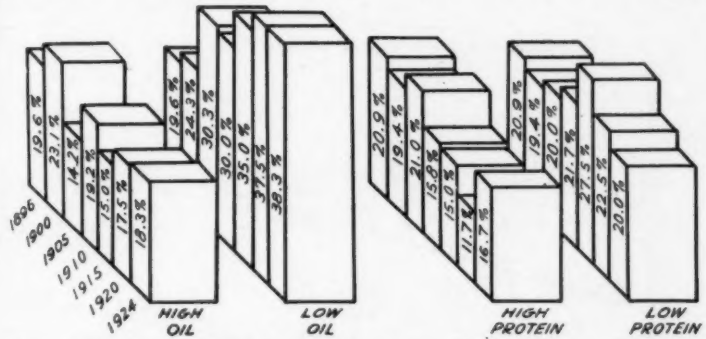

FIGURE 9.—Graphic presentation of the modal classes with respect to the percentage composition of Illinois high and low oil, and high and low protein corn, at periodic intervals between 1896 and 1924

in all cases variability progresses in the same direction as the mean when measured by the standard deviation, Weinberg formula, and the extramodal coefficient. If variability is expressed as a percentage of the mean it decreases as the mean increases, and vice versa. Of the four strains, the low protein shows on an average the least change in variability. (Table 2.)

RELATIVE VARIABILITY IN PROTEIN AND OIL

The protein strains show greater variability than do the oil strains (Tables 3–6), thus suggesting that the former may be affected to a greater extent by the environment, Hopkins states (*8 p. 239*) "the fat content of corn is even more susceptible to the influence of seed selection than is the protein content, doubtless due to the fact that the primary materials from which fat is manufactured, namely, carbon dioxide and water, are usually furnished to the plant in unlimited supply, while the formation of protein is essentially dependent upon the supply of available nitrogen in the soil." Variability is usually augmented when the living material under study is grown at the minimum or maximum rather than at the optimum condition.

TABLE 2.—*Comparison of change in variability for chemical composition in four Illinois strains of corn as determined by the best fitting lines for four different measures of variability, 1896 and 1924*

Strain	Method used to measure variability	Year		Percentage of increase (+) or decrease (−)
		1896	1924	
High protein	Coefficient of variation	10.03	9.54	[a] −4.86
	Standard deviation	1.18	1.57	+33.05
	Weinberg's formula	.95	1.19	+25.26
	Extramodal coefficient	3.79	6.37	+68.07
Low protein	Coefficient of variation	10.53	12.02	+14.15
	Standard deviation	1.01	.86	−14.85
	Weinberg's formula	.89	.86	−3.37
	Extramodal coefficient	4.19	3.14	−25.06
High oil	Coefficient of variation	8.11	6.23	−23.18
	Standard deviation	.43	.64	+48.84
	Weinberg's formula	.59	.73	+23.73
	Extramodal coefficient	4.09	5.02	+22.74
Low oil	Coefficient of variation	8.50	14.44	+69.88
	Standard deviation	.34	.21	−38.24
	Weinberg's formula	.56	.39	−30.36
	Extramodal coefficient	3.68	1.50	−59.24

[a] These figures differ slightly from those in the text. The figures in the text were based on four figures after the decimal.

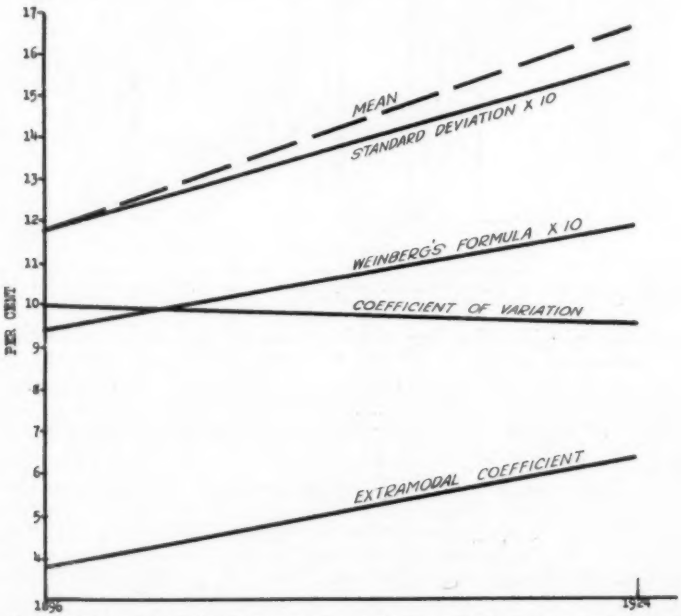

FIGURE 10.—Comparison of four different measures of variation for protein content of Illinois high-protein corn as expressed by the best fitting straight lines; the broken line shows the trend of the mean protein content

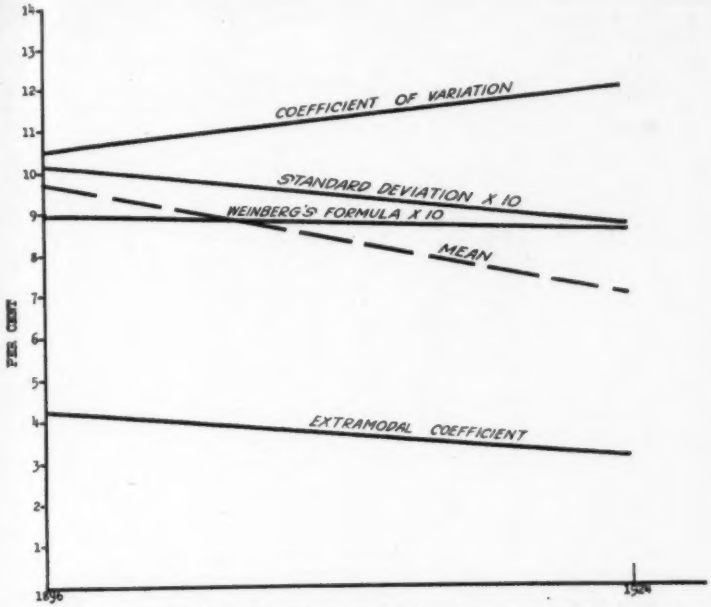

FIGURE 11.—Comparison of four different measures of variation for protein content of Illinois low-protein corn as expressed by the best fitting straight lines: the broken line shows the trend of the mean protein content

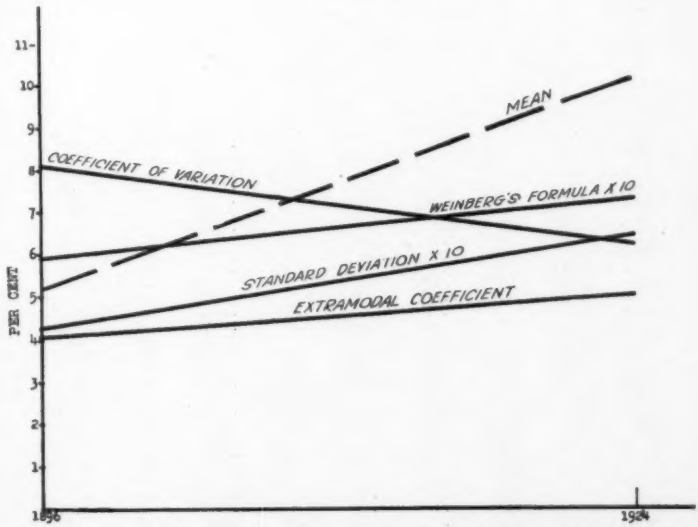

FIGURE 12.—Comparison of four different measures of variation for oil content of Illinois high-oil corn as expressed by the best fitting lines; the broken line shows the trend of the mean oil content

TABLE 3.—*Average protein content and its percentage variability in Illinois high-protein strain corn, 1896–1924, as measured by different methods, together with extreme variates*

Year	Ears analyzed	Average protein content	Standard deviation	Coefficient of variation	Variability by Weinberg's formula	Lowest variate	Highest variate	Population in modal class
	Number	*Per cent*			*Per cent*			*Per cent*
1896	163	* 10.93	* 1.04	* 9.50	0.881	8.3	13.9	20.9
1897	112	10.99	1.16	10.90	1.008	8.3	13.6	.....
1898	252	10.98	1.22	11.15	.912	7.7	14.9	.....
1899	216	11.62	1.28	11.00	1.012	8.4	14.8	.....
1900	216	12.62	1.02	8.09	.795	9.3	15.7	19.4
1901	114	13.78	1.17	8.48	1.103	11.5	16.0	.....
1902	90	12.90	1.10	8.50	.965	9.5	15.0	.....
1903	100	13.51	1.36	10.04	.925	8.5	17.3	.....
1904	100	15.03	1.36	9.05	1.040	10.6	17.8	.....
1905	119	14.73	1.26	8.55	.998	10.8	17.4	21.0
1906	120	14.26	1.31	9.19	.978	10.5	17.7	.....
1907	120	13.90	1.49	10.72	1.119	10.3	17.4	.....
1908	119	13.94	1.66	11.91	1.193	9.4	17.3	.....
1909	120	13.29	1.45	10.76	1.076	9.2	16.4	.....
1910	120	14.87	1.44	9.68	1.109	11.2	18.0	15.8
1911	120	13.79	1.79	12.98	1.344	10.3	17.4	.....
1912	120	14.49	1.13	7.80	.854	10.3	17.5	.....
1913	120	14.83	1.22	8.23	.965	11.6	18.0	.....
1914	120	15.04	1.42	9.44	1.148	11.5	17.8	.....
1915	120	14.54	1.47	10.19	1.076	10.7	18.2	15.0
1916	120	15.66	1.34	8.56	1.047	12.7	19.3	.....
1917	120	14.45	1.85	12.80	1.277	10.2	18.6	.....
1918	120	15.49	1.36	8.78	1.022	11.8	18.9	.....
1919	120	14.70	1.55	10.54	1.201	11.1	17.8	.....
1920	120	14.01	1.79	12.78	1.287	9.5	17.4	11.7
1921	120	16.66	1.84	11.04	1.441	9.4	18.8	.....
1922	120	17.34	1.24	7.15	.890	12.6	20.6	.....
1923	120	16.53	1.41	8.50	1.116	13.1	19.7	.....
1924	120	16.60	1.19	7.17	1.120	14.6	19.2	16.7

* Data for the years 1896–1903 are taken from Davenport's Principles of Breeding (*1, p. 446*) means and standard deviations for the remaining years are calculated by the nongrouping method.

TABLE 4.—*Average protein content and its percentage variability in Illinois low-protein strain corn, 1896–1924, as measured by different methods, together with extreme variates*

Year	Ears analyzed	Average protein content	Standard deviation	Coefficient of variation	Variability by Weinberg's formula	Lowest variate	Highest variate	Population in modal class
	Number	*Per cent*			*Per cent*			*Per cent*
1896	163	* 10.93	* 1.04	* 9.50	0.881	8.3	13.9	20.9
1897	60	10.63	.90	8.47	.759	8.2	14.0	.....
1898	126	10.49	1.32	12.61	1.089	7.5	13.4	.....
1899	144	9.59	1.01	10.50	.802	6.7	13.1	.....
1900	144	9.13	1.04	11.34	.937	7.1	12.3	19.4
1901	126	9.63	1.10	11.47	.975	7.6	13.1	.....
1902	90	7.86	.75	9.60	.829	6.4	9.7	.....
1903	100	8.00	.83	10.41	.862	6.4	10.2	.....
1904	100	8.17	.81	9.91	.773	6.1	10.5	.....
1905	120	8.58	.85	9.91	.753	6.6	12.1	20.0
1906	120	8.65	.92	10.64	.899	6.7	10.9	.....
1907	120	7.32	.92	12.57	.910	5.8	10.5	.....
1908	120	8.96	1.26	14.06	1.117	6.3	11.4	.....
1909	120	7.48	.94	12.57	.842	5.5	10.8	.....
1910	120	8.26	.86	10.41	.827	6.5	11.0	21.7
1911	120	7.90	1.17	14.81	1.005	5.9	12.1	.....
1912	120	8.23	.82	9.96	8.860	6.8	10.8	.....
1913	120	7.71	.95	12.32	.861	5.7	10.8	.....
1914	120	7.67	.95	12.39	.890	5.9	10.8	.....
1915	120	7.27	.85	11.69	.854	5.7	9.9	27.5
1916	120	8.68	1.03	11.86	.994	6.6	10.9	.....
1917	120	7.09	.71	10.01	.233	5.6	9.6	.....
1918	120	7.13	.75	10.52	.894	5.9	8.8	.....
1919	120	6.46	.52	8.05	.629	5.4	8.3	.....
1920	120	7.54	.89	11.80	.890	6.0	10.5	22.5
1921	120	9.14	1.35	14.77	1.074	6.6	13.4	.....
1922	120	7.42	.70	9.43	.774	6.1	9.6	.....
1923	120	6.48	.73	11.27	.708	5.0	9.4	.....
1924	120	8.38	1.17	13.96	.998	6.1	11.8	20.0

* Data for the years 1896, 1898, 1899, 1900, 1901, 1902, and 1903 are taken from Davenport's Principles of Breeding (*1, p. 446*); means and standard deviations for the remaining years are calculated by the nongrouping method.

TABLE 5.—*Average oil content and its percentage variability in Illinois high-oil strain corn, 1896–1924, as measured by different methods, together with extreme variates*

Year	Ears analyzed	Average oil content	Standard deviation	Coefficient of variation	Variability by Weinberg's formula	Lowest variate	Highest variate	Population in modal class
	Number	*Per cent*			*Per cent*			*Per cent*
1896	163	* 4.68	* 0.41	* 8.83	0.585	3.9	6.0	19.6
1897	80	4.79	.38	7.87	.543	3.6	5.7	
1898	216	5.10	.48	9.33	.615	4.1	6.7	
1899	108	5.65	.42	7.47	.582	4.3	6.5	
1900	108	6.10	.44	7.26	.527	4.6	7.4	23.1
1901	126	6.24	.45	7.26	.621	4.9	7.1	
1902	90	6.25	.50	8.06	.680	5.0	7.2	
1903	100	6.51	.46	7.07	.608	5.5	7.6	
1904	101	7.11	.61	8.61	.792	6.0	8.4	
1905	120	7.30	.49	6.70	.651	6.3	8.6	14.2
1906	120	* 7.38	.40	5.46	.594	6.6	8.5	
1907	120	7.43	.51	6.82	.522	6.2	8.7	
1908	120	7.21	.53	7.36	.658	5.9	8.5	
1909	120	7.05	.47	6.62	.580	5.8	8.4	
1910	120	7.72	.57	7.36	.718	6.5	9.0	19.2
1911	120	7.52	.69	9.20	.763	5.9	9.2	
1912	120	7.71	.50	6.49	.778	6.5	8.7	
1913	120	8.16	.62	7.63	.700	6.4	9.6	
1914	120	8.30	.68	8.23	.608	5.8	10.1	
1915	120	8.47	.49	5.77	.576	6.9	9.8	15.0
1916	120	8.51	.49	5.80	.592	7.2	10.0	
1917	120	8.52	.58	6.78	.762	7.2	9.7	
1918	120	9.36	.53	5.67	.674	8.0	10.5	
1919	120	9.06	.84	9.25	.961	7.3	10.4	
1920	120	9.28	.52	5.57	.619	7.8	10.6	17.5
1921	120	9.94	.66	6.64	.729	8.4	11.7	
1922	120	9.86	.54	5.48	.674	8.7	11.3	
1923	120	10.08	.65	6.45	.695	8.3	11.8	
1924	120	9.86	.61	6.19	.676	8.4	11.7	18.3

* Data for the years 1896–1903 are taken from Davenport's principles of breeding (*1, p. 446*); means and standard deviations for the remaining years are calculated by the nongrouping method.

TABLE 6.—*Average oil content and its percentage variability in Illinois low-oil strain corn, 1896–1924, as measured by different methods, together with extreme variates*

Year	Ears analyzed	Average oil content	Standard deviation	Coefficient of variation	Variability by Weinberg's formula	Lowest variate	Highest variate	Population in modal class
	Number	*Per cent*			*Per cent*			*Per cent*
1896	163	* 4.68	* 0.41	* 8.83	0.585	3.9	6.0	19.6
1897	50	4.10	.29	7.10	.510	3.4	4.7	
1898	108	3.59	.32	8.13	.389	3.2	4.8	
1899	144	3.85	.32	8.42	.484	2.8	4.6	
1900	144	3.57	.36	10.13	.522	2.6	4.5	24.4
1901	126	3.45	.26	7.59	.456	2.8	4.1	
1902	90	3.00	.32	10.83	.492	2.1	3.8	
1903	90	2.99	.23	7.83	.441	2.5	3.6	
1904	100	2.89	.46	15.91	.920	2.4	3.4	
1905	119	2.58	.31	11.86	.602	1.8	3.1	30.3
1906	120	2.67	.30	11.35	.443	1.6	3.5	
1907	120	2.60	.24	9.04	.466	2.2	3.3	
1908	120	2.39	.27	11.09	.507	1.8	2.9	
1909	120	2.24	.23	10.40	.402	1.4	2.8	
1910	120	2.21	.20	9.05	.384	1.6	2.7	30.0
1911	120	2.06	.23	11.02	.398	1.4	2.7	
1912	120	2.19	.22	10.05	.386	1.3	2.7	
1913	120	1.91	.24	12.30	.451	1.3	2.4	
1914	120	1.98	.26	13.33	.446	1.3	2.7	
1915	120	2.07	.25	11.93	.410	1.4	3.1	35.0
1916	120	2.07	.50	24.30	.828	1.3	4.7	
1917	120	2.10	.24	11.38	.474	1.7	2.8	
1918	120	1.88	.32	16.86	.489	1.2	3.0	
1919	120	1.77	.21	11.92	.394	1.3	2.5	
1920	120	1.80	.21	11.83	.323	1.0	2.4	37.5
1921	120	1.71	.15	8.77	.264	1.0	2.3	
1922	120	1.68	.19	11.55	.347	.9	2.2	
1923	120	1.58	.24	15.19	.480	1.1	2.1	
1924	120	1.51	.22	14.57	.387	.9	2.2	38.3

* Data for the years 1896, 1898, 1899, 1900, 1901, 1902, and 1903 are taken from Davenport's Principles of Breeding (*1, p. 446*); means and standard deviations for the remaining years are calculated by the nongrouping method.

SYMMETRY OF DISTRIBUTION

A number of other statistical expressions have been proposed intended to describe the nature of a population with respect to its distribution. Certain of these expressions are of interest in connection with the present study. An important item of information in regard to a distribution is whether the variates are symmetrically distributed with reference to the mean or whether there is a bunching of variates on one side of the mean and a long tailing out of the variates on the other side; i. e., to know the amount of skewness.

Another item that should be known is whether the variates are densely grouped at the mean, giving a high peak to the frequency

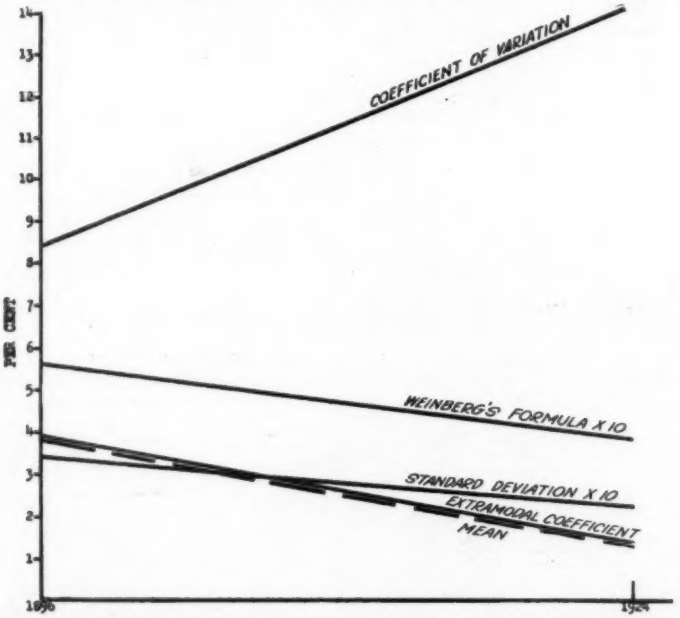

FIGURE 13.—Comparison of four different measures of variation for oil content of Illinois low-oil corn as expressed by the best fitting straight lines; the broken line shows the trend of the mean oil content

polygon, or whether the distribution is rather flat in the middle and contracted at the ends, or whether the distribution of the variates is intermediate between these two conditions; i. e., to know the amount of kurtosis. A normal distribution is said to be mesokurtic, a peaked curve leptokurtic, and a flat curve platykurtic.

The mean, median, mode, standard deviation, coefficient of variation, variation by Weinberg's formula, skewness, and kurtosis are given in Table 7 for each of the four strains taken at periodic intervals. The median, mode,[4] skewness,[5] and kurtosis[6] were calculated

[4] Mode=Mean−3.03 (mean−median).

[5] Skewness=$P_{.50}-\frac{1}{2}D$. S. D. of Sk=0.50914 $\frac{D}{\sqrt{N}}$. $D=P_{.50}-P_{.10}$.

[6] Kurtosis=$\frac{Q}{D}$. Q=quartile deviation. S. D. of Ku.=$\frac{0.27779}{\sqrt{N}}$.

on the basis of percentiles.[7] Kelley (*10, p. 58–62, 75–77*) states that "this method of determining curve types, although in general not as accurate as the longer method of Pearson, can be used where the populations are large and the standard errors are small." The variates of the high-oil and the low-oil strains appear to be fairly symmetrically distributed with reference to the mean. In no case is skewness significantly different from zero. In five of the seven years studied the median is greater than the mean in the high-oil strain. In the low-oil strain the median is lower than the mean in four of the seven years. The differences in all cases are very small.

TABLE 7.—*Measurements of central or average tendencies, and of dispersions for distributions of the variates of four Illinois strains of corn taken at periodic intervals*

Year	Number of ears analyzed	Mean	Median	Mode	Standard deviation [a]	Coefficient of variation [a]	Variability by Weinberg's formula [a]	Skewness [b]	Kurtosis [c]
HIGH-PROTEIN STRAIN									
1896	163	10.96	11.04	11.18	1.04	9.50	0.88	+0.1082±0.0874	−0.0008±0.0147
1900	216	12.69	12.64	12.55	1.02	8.09	.80	−.1312± .0704	+.0101± .0127
1905	119	14.77	14.94	15.29	1.26	8.55	1.00	+.3997± .2312	−.0320± .0172
1910	120	14.93	15.08	15.37	1.44	9.68	1.11	+.2159± .1313	+.0229± .0171
1915	120	14.55	14.67	14.92	1.47	10.19	1.08	+.2447± .1426	+.0106± .0171
1920	120	14.04	13.92	13.67	1.79	12.78	1.29	−.2885± .1775	+.0206± .0171
1924	120	16.68	16.65	16.58	1.19	7.17	1.12	−.1027± .1237	−.0065± .0171
LOW-PROTEIN STRAIN									
1896	163	10.96	11.04	11.18	1.04	9.50	0.88	+0.1082±0.0874	+0.0008±0.0147
1900	144	9.19	9.07	8.83	1.04	11.34	.94	−.2044± .0922	+.0043± .0156
1905	120	8.64	8.48	8.15	.85	9.91	.75	−.1772± .0946	+.0398± .0171
1910	120	8.32	8.24	8.08	.86	10.41	.83	−.1443± .0870	−.0085± .0171
1915	120	7.30	7.16	6.85	.85	11.69	.85	−.2182± .0775	+.0018± .0171
1920	120	7.60	7.50	7.29	.89	11.80	.89	−.1543± .0835	+.0061± .0171
1924	120	8.44	8.34	8.14	1.17	13.96	1.00	−.1865± .1049	−.0050± .0171
HIGH-OIL STRAIN									
1896	163	4.74	4.83	5.00	0.41	8.83	0.59	+0.0323±0.0391	−0.0368±0.0147
1900	108	6.15	6.20	6.31	.44	7.26	.53	+.0654± .0653	−.0051± .0180
1905	120	7.35	7.34	7.32	.49	6.70	.65	−.0053± .0519	+.0330± .0171
1910	120	7.78	7.72	7.60	.57	7.36	.72	−.1090± .0507	−.0274± .0171
1915	120	8.52	8.58	8.69	.49	5.77	.58	+.1098± .0574	+.0014+ .0171
1920	120	9.34	9.35	9.38	.52	5.57	.62	+.0850± .0491	−.0192± .0171
1924	120	9.92	10.00	10.17	.61	6.19	.68	+.0117± .0726	−.0301± .0171
LOW-OIL STRAIN									
1896	163	4.74	4.83	5.00	0.41	8.83	0.59	+0.0323±0.0391	−0.0368±0.0147
1900	144	3.63	3.61	3.56	.36	10.13	.52	−.0565+ .0304	+.0007± .0152
1905	119	2.64	2.62	2.58	.31	11.86	.50	−.0197± .0262	+.0250± .0172
1910	120	2.26	2.27	2.29	.20	9.05	.38	+.0260+ .0241	+.0171± .0171
1915	120	2.13	2.12	2.12	.25	11.93	.41	−.0177± .0209	+.0155± .0171
1920	120	1.84	1.86	1.89	.21	11.83	.32	+.0257± .0227	−.0268± .0171
1924	120	1.56	1.54	1.52	.22	14.57	.39	−.0130± .0240	−.0322± .0171

[a] Taken from Tables 3, 4, 5, and 6.

[b] A positive sign (+) indicates that the distribution tails out on the low side.; a negative sign (−) indicates the reverse.

[c] Data reported equals Ku. less 0.26315. If sign is positive (+) curve is platykurtic; if sign is negative (−) curve is leptokurtic.

[7] Determine the class in which the $pN+\frac{1}{2}$ measure lies.
Let f_p=the frequency in this class.
Let i_p=the interval or range covered by this class.
Let F_p=the sum of the frequencies in all classes below this class.
Let v_p=the value of the lower boundary of this class.
Let N=the total population.
Let P_p=percentile, the value of which is to be calculated.
Let p=proportion of classes having values smaller than P_p.
Then $P_p=v_p+\frac{pN-F_p}{f_p}i_p$.

The distributions for the low-protein strain are negatively skewed, i. e., tail out on the high side for each of the years studied except 1896. Although the skewness is much greater than in the case of the oil strains, in no instance is skewness significantly different from zero. Likewise, the distributions for the high-protein strain show a greater skewness than do the distributions for the oil strains, but in no case is the skewness significantly different from zero.

The four strains show but little deviation from a mesokurtic curve. The small deviations from the normal probability curve are as frequent in the direction of a leptokurtic curve as of a platykurtic curve.

A study of the extreme variates of the different strains for the period of selection (fig. 14) shows that in the high-oil strain the variates deviate as far on the low side as they do on the high side. In the low-oil strain the deviation is slightly greater on the high side.

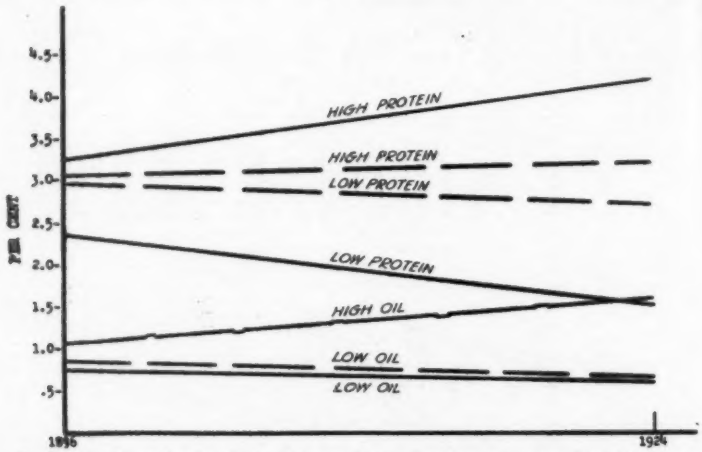

FIGURE 14.—Comparison of the range between the lowest variate and the mean (solid line), and the highest variate and the mean (broken line), for high-protein, low-protein, high-oil, and low-oil Illinois corn as expressed by the best fitting straight lines

However, the extreme variates in the high-oil strain are about twice as far from the mean as those in the low-oil strain. The range in the low-oil strain is becoming less while that in the high-oil strain is becoming greater.

The extreme variates in the low-protein strain are farther from the mean in a positive direction than in a negative direction, while in the high-protein strain the reverse is true. This means that the distribution tails out toward the mean of the original nonselected material in both cases.

GENERAL DISCUSSION

If, as has been stated by many, the population with the greatest variability offers the greatest chance for improvement by selection then it should be possible to make still greater progress in the Illinois high-protein and Illinois high-oil strains in future years than has been made in the past because the range of each was greater in 1924

than it was in 1896. (Fig. 15.) Such a conclusion would be rather questionable. As has been stated, the strains now appear to be more nearly homozygous than was the original material, and our knowledge of genetics and the effects of selection would lead us to believe that they should be. Being more nearly homozygous, they should be less variable genetically. The apparent increase in variability must then be due either to the environment or to the methods of measuring variability.

Emerson (*4, p. 30–31*) in speaking of the variability in number of rows on an ear of corn says: ". . . It is more reasonable to suppose

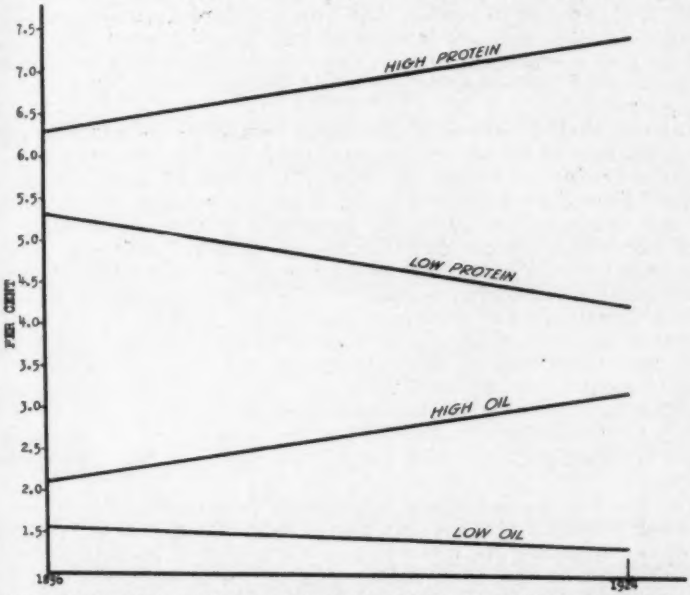

FIGURE 15.—Comparison of the ranges between the highest variate and the lowest variate for high-protein, low-protein, high-oil, and low-oil Illinois corn as expressed by the best fitting straight lines

that an ear which can vary in any one of eight spikes will show a greater degree of fluctuation than one which can vary only in any one of four spikes. For this reason it is likely that strains with a high number of rows will never show the low variability seen in strains with a low number of rows." One may reason in like manner about variability of the oil and of the protein content for the different strains. The high strains are more variable than the low strains because there is more material present for the environment to interact with. Supposing the germ of the high-oil strain to contain 600 cells and that of the low oil to contain 300 cells, we might expect twice the effect on the high-oil strain from environmental action as on the low oil under identical conditions.

Zeleny (*15, p. 15*) states: "It is a common principle of embryology that a changed condition does not act by accretion, i. e., by addition

or subtraction of individual parts without affecting the rest. On the contrary, the action is upon all of the preexisting parts of the organ." This he calls the theory of proportionate action. Zeleny, in the study of the effect of selection for eye facet number in the white bar-eye race of *Drosophila melanogaster*, found that a race with 300 facets was affected ten times as much as a race with only 30 facets by a 1° change in temperature. In order to measure the variability of races with different facet numbers he scaled his classes for grouping so that each class range was a fixed percentage of the mean of its class. He used as a mid or zero point the mean of the original population from which his selected material came. He (*15, p. 15*) goes on to explain that "the standard deviation, as determined by this method, is expressed in factorial units and serves directly as a coefficient of variation strictly comparable in all cases, regardless of the mean values of the different stocks that may be compared."

Should such a method of measuring variability be applied to the distributions of the oil and protein strains in the present study, it can be readily seen that the degree of deviation from the means would depend upon the value taken as a percentage of the means of the classes. The larger this figure the smaller the deviation of the high strain as compared to the low strain. Also, the farther the high strain is removed from the low strain the greater will be its comparative reduction in variability. The factorial method of measuring variability has not been used in an analysis of these data because the method affords no manner of determining the figure to be used in arriving at the class ranges with such data. Such a figure might be 10, 20, or 23 per cent. Zeleny apparently uses such a figure as will give a normal distribution for the populations studied. As has been shown, the distributions for protein and for oil content thus far studied do not show a significant deviation from the normal curve.

Although it appears logical to attribute the increase in variation in the high strains to the fact that there is more material for the environment to interact with, it is impossible to prove it, because the variability due to the environment can not be separated from the variability due to the segregation and recombination of factors. The complexity of the inheritance of protein and oil content and the manner of conducting the selection work make it entirely impossible to analyze the four strains genetically. Others who have studied the protein content of corn have also found it impossible to determine the genetic factors involved. East (*3*) lists a large number of factors other than genetic that may and probably do affect protein content, e. g., number of seeds on the ear, size of pericarp, lack of phosphorus, and departure from optimum temperature and moisture at critical periods. In addition to these there may be considered the factors affecting size of germ, size of endosperm, absorption of different amounts of food elements through the roots and their translocation through the stalk and subsequent deposition within the pericarp. The total protein content is made up chemically of at least four protein groups (*13*) each of which may be represented by a single or several genes in the germ plasm. Some of these factors may be dominant and others recessive, or there may be any graduation of dominance. Hayes (*5*) states: "Protein content is therefore inherited in

much the same way as other characters which are dependent for their full expression on many different inherited factors of the plant and likewise upon environmental conditions." What has been said of the protein content may likewise be said of the oil content.

SUMMARY

Twenty-eight years of continuous selection for protein and oil content in corn has produced four types which are distinctly different in their composition. When compared with the original nonselected material the high-protein and the high-oil strains show a proportional increase of 50.01 and 109.79 per cent, respectively. The low-protein and low-oil strains show a proportional decrease of 23.26 and 67.87 per cent, respectively.

The high-protein and high-oil strains show no indications of having reached a limit to further increases.

The low-protein strain has changed but little during the last 20 years.

The low-oil strain is approaching a physiological limit to further decreases. Ears with extremely low oil content have a high percentage of germless seeds.

The four different strains now trace back in their pedigrees to a single ear each.

Variability has been shown to change considerably following selection. The degree of change in variability depends somewhat upon the method used in measuring it.

Variability of oil or protein content appears to depend upon the magnitude of the mean of the selected character.

Variability as measured by the coefficient of variation increases when selection leads to a low mean, and vice versa.

Variability, as measured by the standard deviation, Weinberg's formula, and extramodel coefficient, increases when selection leads to a high mean, and vice versa.

The percentage of the population lying in the model classes decreases when selection leads to a high mean, and vice versa.

The symmetry of the distribution curve as determined by the percentile method for the four strains taken at periodic intervals is not significantly different from that of the normal variability curve.

It is suggested that the apparent increase in variability of the high strains may be due to the fact that there is more material present for the environment to interact with.

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